Endothelin system expression and function in failing and nonfailing myocardium

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Declaration

I hereby declare that the work described in this thesis was performed entirely by myself, except for procedures acknowledged in the text. This work contains no material that has been accepted for the award of any other degree or diploma in any university or tertiary institution and to the best of my knowledge contains no material previously published or written by another person, except where stated in the text.

Alison Fiona Wallace

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Abstract

The peptide endothelin (ET-1) was first described as an endothelium-derived potent vasoconstrictor substance. However, cardiomyocytes can also synthesise ET-1 and express ET_A and ET_B receptors. Previous studies in our laboratory have demonstrated that healthy rat myocardium contains both ET_A (80%) and ET_B receptors (20%) and that expression of the ET_B subtype is increased in rats with chronic heart failure (CHF) following coronary artery ligation (CAL). However, the significance of ET_B upregulation is unknown and functional studies investigating the relative roles of the ET_A and ET_B receptors in normal or failing hearts have proved inconsistent.

The aims of this thesis were to investigate the endothelin system in failing and nonfailing myocardium and to evaluate the role of ET receptor function in relation to contractility *in vivo* and *in vitro*. In addition, transmural differences in gene expression were explored in human failing myocardium to establish whether genes associated with stiffness, hypertrophy and the ET system correlate to wall stress.

Rats underwent myocardial infarction (MI) and developed left ventricular (LV) dysfunction at 15 weeks post-MI, as evidenced by an increase in LV end-diastolic pressure (LVEDP, 3 ± 0.2mmHg to 12 ± 2mmHg, $P<0.01$) and an increase in expression of atrial natriuretic peptide mRNA. A reduction in the basal contractile response of papillary muscles from failing hearts was also consistent with dysfunction. However, responses to ET-1 were inconsistent and prevented conclusions about the role of individual receptors. Investigation of ET receptor expression by RT-PCR failed to show a selective increase in ET_B receptors and prepro ET-1 mRNA remained unchanged at 15 weeks post-MI. Subsequently, experiments were designed to investigate the influence of ET on myocardial contractility using ET receptor antagonists in papillary muscles from control animals. Results suggested that both ET receptors contribute to contractility in rat myocardium, corresponding with the presence of both receptor subtypes, found using RT-PCR. Furthermore, the data showed that expression of ET receptors in the heart

undergo seasonal variation, which corresponds with the contribution of receptors to regulation of contractility.

LV function was evaluated *in vivo* using a novel technique developed in our laboratory. Echocardiography in conjunction with associated LV pressure data demonstrated a reduced shortening fraction (SF, 18.7 ± 1.9% to 13.6 ± 1.3%, $P<0.05$) and ejection fraction (EF, 31.1 ± 2.6% to 22.4 ± 1.2%, $P<0.01$) in rats 12 weeks post-MI indicating LV dysfunction, also evidenced by elevated LVEDP (5.7±1.0mmHg to 15.6±1.5mmHg $P<0.001$). Subsequently, the technique was used on control animals following chronic (7 day) administration of Bosentan, a dual ET receptor antagonist (100mg/kg/day). However, antagonist treatment failed to influence haemodynamics, SF or EF, suggesting that unlike *in vitro*, the ET system does not play a central role in regulating cardiac function *in vivo*.

In the human heart, wall stress is highest in the endocardial layer. Different types of heart failure alter myocardial contractility and wall stress in different ways. Semi-quantitative RT-PCR identified that genes linked to hypertrophic remodelling were consistently increased in the endo relative to epicardium. Interestingly, expression of the ET_A receptor and the cytoskeletal elastic protein, titin, in particular the N2B isoform linked to increased myocardial stiffness showed 2 distinct patterns of expression across the LV wall, depending on the type of heart failure. ET_B expression was uniform across the wall.

In conclusion, the ET system is expressed in the normal myocardium and both receptors can regulate contractility *in vitro*, however the system is not essential for normal heart function *in vivo*. The time course and severity of experimental heart failure influences the expression of ET system in the rat, with results showing that modification of ET system expression does not always accompany LV dysfunction. In the failing human heart, proteins associated with wall stress and hypertrophy are most abundant in the endocardium of the LV, while transmural distribution of the ET_A receptor and the 'giant spring' protein, titin, depends on etiology.

Publications and Presentations

Abstracts and presentations:

Wallace A.F., Denvir M.A. & Gray G.A. (2000) 'Endothelin-1 stimulates tension development in rat isolated right ventricular papillary muscle'. Moderated poster presentation at The British Society for Cardiovascular Research, Belfast, September 2000.

Wallace A.F., Denvir M.A. & Gray G.A. (2000) 'Investigation of myocardial endothelin receptor function in chronic heart failure'. Poster presentation at the 4th Scottish Cardiovascular Forum, Glasgow, January 2001.

Wallace A.F., Denvir M.A. & Gray G.A. (2000) 'Investigation of ventricular endothelin receptors in heart failure'. Poster presentation at the 3rd Wellcome Trust Cardiovascular Research Initiative, Edinburgh, June 2001.

Wallace A.F, Denvir M.A. & Gray G.A. (2001) 'Endogenous ET-1 regulates ventricular muscle contractility via both ET_A and ET_B receptors'. Poster presentation at the Seventh International Conference on Endothelin, Edinburgh, September 2001.

Wallace A.F., Denvir M.A. & Gray G.A. (2002) 'Seasonal variation of myocardial endothelin receptor mRNA expression in rats'. Moderated poster presentation at the British Pharmacology Society, Glasgow, September 2002.

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Millar C.A., Ye P., Gray G.A., Wallace A.F., MacKenzie S.M., Fraser R., Connell J.M.C. & Davies E. (2003) 'Aldosterone synthase (CYP11B2) and 11beta-hydroxylase (CYP11B1) gene expression in the normal and failing rat heart'. British Endocrine Society, Spring 2003.

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Papers in preparation:

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List of selected abbreviations and acronyms

AC	Adenylyl cyclase
ACE	Angiotensin converting enzyme
Ang II	Angiotensin II
ANP	Atrial natriuretic peptide
AV	Atrioventricular
BDM	2,3-Butanedione-monoxime
BNP	Brain natriuretic peptide
BW	Body weight
Ca^{2+}	Calcium ion
CAL	Coronary artery ligation
cAMP	cyclic 3',5' -adenosine monophosphate
cGMP	cyclic 3',5'-guanosine monophosphate
CHF	Chronic heart failure
CSA	Cross sectional area
DAG	1,2-Diacylglycerol
DCM	Dilated cardiomyopathy
DNA	Deoxyribonucleic acid
ECE	Endothelin converting enzyme
ESPDR	End-systolic pressure-dimension relationship
ET-1	Endothelin-1
ET_A	Endothelin A receptor
ET_B	Endothelin B receptor
GAPDH	Glyceraldehyde-3-phosphate
HCM	Hypertrophic cardiomyopathy
HR	Heart rate
IHD	Ischaemic heart disease
IP_3	Inositol 1,4,5-triphosphate
LA	Left atrium
LV	Left ventricle
LVD	Left ventricular dysfunction
LVEDP	Left ventricular end-diastolic pressure

LVSP	Left ventricular systolic pressure
LVH	Left ventricular hypertrophy
MAP	Mean arterial pressure
MAPK	Mitogen-activated protein kinase
MI	Myocardial infarction
mRNA	Messenger ribose nucleic acid
Na^+	Sodium ion
NO	Nitric oxide
NOS	Nitric oxide synthase
PG	Prostaglandin
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
ppET-1	Prepro endothelin-1
RA	Right atrium
RAAS	Renin-angiotensin-aldosterone system
RNA	Ribonucleic acid
RT_{50}	Time from peak to 50% relaxation
RT-PCR	Reverse transcriptase polymerase chain reaction
RV	Right ventricle
SA	Sino-atrial
SEM	Standard error of the mean
SERCA	Sarcoplasmic reticulum Ca^{2+}-ATPase
SMC	Smooth muscles cells
SR	Sarcoplasmic reticulum
SRTX	Sarafotoxin
TPT	Time to peak tension

Chapter 1

Introduction

1.1 The cardiovascular system

The cardiovascular system comprises the heart and two networks of blood vessels, the systemic circulation and the pulmonary circulation, which convey oxygen and nutrients to the tissues of the body and remove metabolic waste products such as carbon dioxide and urea.

1.1.1 Anatomy of the heart

The heart consists of two intermittent muscular pumps, the right and left ventricles, which are filled from reservoirs, the right and left atria. The heart wall is made up of three layers, the endocardium, myocardium and epicardium, each with particular structure and function (as reviewed by Stevens & Lowe, 1992). A delicate connective-tissue membrane lined with endothelium comprises the innermost layer of the endocardium. This forms a complete lining for the atrial and ventricular cavities, it covers all the structures that project into the heart (valves, chordae tendineae and papillary muscles) and is continuous with the lining of the major vessels. Endothelial cells within the heart release a number of substances that modulate myocardial contractile function. These agents include nitric oxide (NO), endothelin-1 (ET-1), prostanoids, adenylpurines and other substances, which are likely to be important mechanisms both physiologically and in pathological states (as reviewed by Shah *et al.*, 1996; Brutsaert, 2003). The middle layer of endocardium is the thickest and consists of dense connective tissue along with elastic fibres that alternate with layers of collagenic fibres, while the outer layer is composed of collagen fibres that are arranged more irregularly and merge with collagen surrounding adjacent cardiac muscle fibres. This layer has blood vessels and Purkinje fibres and may also contain fat.

The myocardium is the thickest part of the wall and is responsible for contraction. It is comprised of specialised cardiac muscle fibres that form an interconnecting network (syncytium) separated by loose fibrocollagenous tissue termed the endomysium. It also contains conducting cells and vascular and connective tissue. The left and right atria have thin myocardial layers as they expel blood against

minimal resistance. The right ventricle (RV) has a moderate muscle layer as it has to push blood through the pulmonary valve and into the pulmonary tree. The thickest myocardial layer is observed in the left ventricle (LV), as it has to pump blood against the high resistance of the systemic circulation.

The epicardium is the third outermost layer of the heart, which varies over different chambers. The superficial layer consists of ordinary connective tissue, which is covered by mesothelium and contains some capillaries and nerves. Larger blood vessels and more fat are contained in the deeper layer, which is continuous with the endomysium of underlying myocardium. A mesothelial-lined fibro-elastic membrane termed the pericardium also surrounds the heart and separates it from the rest of the thoracic viscera. Between this and the epicardium is a cavity filled with fluid to lubricate and allow the heart to contract with minimal friction.

The arterial supply of the heart is provided by the left and right coronary arteries, which arise from the aorta, immediately above the aortic valve and lie on the surface of the heart. Smaller branches then branch off from the surface to penetrate into the myocardium. The right coronary artery supplies the right atrium (RA) and RV and also serves both ventricles via the posterior intraventricular branch. The left coronary artery is larger and divides into an anterior interventricular branch (left anterior descending artery), which serves the LV, RV and the interventricular septum, and a circumflex branch, which serves the left atria (LA) and LV lateral and inferior walls.

Valves between the atrium and ventricles, the atrioventricular valves (AV valves), prevent backflow of blood to the atria during systole. The semilunar valves, between the RV and the pulmonary artery or the LV and the aorta, prevent backflow into the ventricles during diastole. Papillary muscles are attached to the cusps of the AV valves by chordae tendineae and contract when the ventricular wall contracts. However, they do not function to help the valves close but rather they pull the valves towards the ventricles to prevent the cusps from being forced into the atrium and turning inside out as the ventricular pressure rises. The chordae tendineae of each papillary muscle are connected to the adjacent parts of two cusps to assist this process (as reviewed by Guyton & Hall, 1996).

1.1.2 Ultrastructure of the myocyte

The architecture and stiffness of the myocardium is preserved by a collagen network, which is found in the extracellular space. Fibrillar collagens, mainly type I and III are coexpressed in a constant ratio (Chapman *et al.*, 1990). This is important to the mechanical properties of tissue because it builds a scaffold to keep the different cellular and noncellular compartments in order and groups the muscle fibres into bundles. Muscle fibres are composed of cardiomyocytes that branch and attach to adjacent cells end-to-end at specialised junctions termed intercalated discs. These connections contain smaller specialised junctions, desmosomes and gap junctions. Desmosomes hold the adjacent cells together, while communicating gap junctions are regions of close apposition to adjacent cell membranes. The gap is spanned by connexin proteins, which form a central pore, allowing the transmission of impulses from cell to cell. The myocyte itself is packed with long contractile bundles of myofibril-like units each composed of smaller units called sarcomeres.

The surface membrane of the bundled myofibril-like units is called the sarcolemma, which is invaginated opposite the Z-line into a series of fine transverse tubules (T-tubules), which run into the cell interior. These structures transmit the electrical stimulus rapidly to the interior of the cell, inducing activation of numerous myofibrils almost simultaneously. A separate tubular system, termed the sarcoplasmic reticulum (SR) exists within the myocyte. It consists of a closed set of anastomosing tubules coursing over the myofibrils and contains a store of Ca^{2+} ions.

The sarcomere is the basic contractile unit of striated muscle and contains many different classes of proteins, assembled into a supramolecular structure with almost crystaline order. They are defined as the material between two Z-lines and align across the cell, giving the muscle its characteristic striated appearance. The sarcomere is composed of two sliding, interdigitating filaments, a thick filament of myosin and a thin filament composed chiefly of actin. Actin filaments are anchored in the Z-line and protrude into the A band, between the myosin rods (*Figure 1.1*). The myocardium develops active force by the cyclic attachment and detachment of myosin crossbridges to actin filaments. The sarcomere shortens when activated by

the binding of Ca^{2+} to troponin C, a component of the troponin complex. This displaces tropomyosin, exposing a myosin-binding site and allows myosin to bind to the thin actin filament. The force of contraction depends on the number of crossbridges formed, which depends directly on the concentration of free Ca^{2+}.

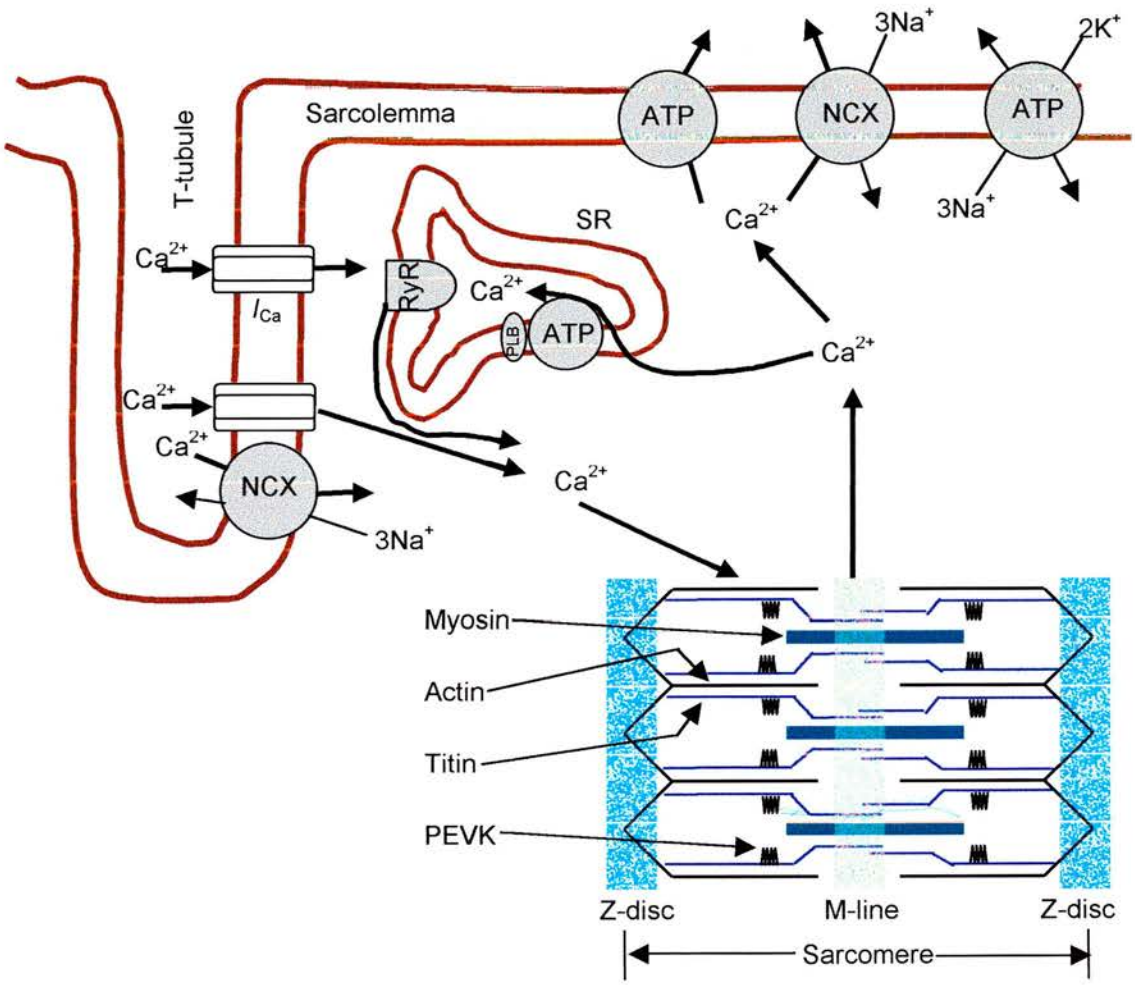


Figure 1.1. Architecture of the myocyte. ATP, ATPase pump; I_{Ca}, inward Ca^{2+} current; NCX, Na^{+}/Ca^{2+} exchange; PLB, phospholambam; RyR, ryanodine receptor; SR, sarcoplasmic reticulum; PEVK, region rich with P (proline), E (glutamate), V (valine) and K (lysine). Modified from Bers (2002) and Gregorio et al. (1999).

The third most abundant cytoskeletal protein is the giant molecule titin, which contributes to the filament system in skeletal and cardiac muscle cells by connecting the Z disc and the central M line of the sarcomere (*Figure 1.1*). Titin provides an extensible scaffold for the contractile machinery and is crucial for myofibrillar elasticity and integrity. In the A band region, titin filaments are composed of regular domains that bind to other proteins of the thick filament (Labeit *et al.*, 1992; Soteroiu *et al.*, 1993). The portion of titin that spans the I band segment is extensible and is likely to account for the intrinsic elasticity of the myofibril and contribute to the diastolic passive tension of striated muscle (see Chapter 6 for titin domain arrangements). Previous studies of titin behaviour have used gelsolin to remove cytoskeletal actin from cardiac myocytes (Granzier *et al.*, 1997) (see Chapter 6). Gelsolin rapidly dissolves actin by severing, capping and nucleating growth and in normal physiology plays an important role in the regulation of filament assembly (as reviewed by Dos Remedios *et al.*, 2003).

1.1.3 Excitation-contraction coupling

Spontaneous rhythmical contraction of the heart is generated and conducted by a specialised system. Impulses originate in the sino-atrial (SA) node, a small strip of pacemaker cells, located in the superior lateral wall of the RA. Any action potential spreads immediately from the node to the atrial tissue and is subsequently conducted to the AV node via internode pathways. A delay allows the atria to contract and empty before ventricular contraction begins. Purkinje fibres then conduct the impulses to the apex of the heart, dividing into smaller branches that course around each ventricular chamber. Rapid transmission of action potentials by Purkinje fibres produces synchronous contraction of the ventricles and therefore effective pumping.

When the cardiac action potential reaches the myocyte, depolarisation causes the opening of fast sodium (Na^+) channels as well as slow Ca^{2+} channels. The ubiquitous second messenger Ca^{2+} is essential for cardiac electrical activity and is the direct activator of the myofilaments. Ca^{2+} enters the cell during the action potential through depolarisation-activated Ca^{2+} channels as inward Ca^{2+} current (I_{Ca}), two classes of voltage-dependent Ca^{2+} channels (L- and T-type) are exhibited on the myocyte.

Entry of Ca^{2+} triggers the release of more Ca^{2+}, termed the Ca^{2+} spark (Cheng *et al.*, 1993), from intracellular stores via SR Ca^{2+} release channels or ryanodine receptors (*Figure 1.1*) and this is known as the Ca^{2+}-induced Ca^{2+}-release mechanism. Concentration of free intracellular Ca^{2+} ($[Ca^{2+}]_i$) is raised by the combined Ca^{2+} influx and SR release, initiating contraction by allowing Ca^{2+} to bind to the myofilament protein troponin C (as reviewed by Bers, 2002). Contraction ends when $[Ca^{2+}]_i$ is reduced. Several routes achieve the removal of free cytosolic Ca^{2+}, most is moved into the SR by the SR Ca^{2+}-ATPase pump, ready for the next contraction. The Na^+/Ca^{2+} exchanger removes some back across the cardiac membrane, while the sarcolemmal Ca^{2+}-ATPase pump and the mitochondrial Ca^{2+} uniporter remove a minimal amount (Bers *et al.*, 1996). Normally the amount of Ca^{2+} removed from the cell during relaxation is the same as the amount that enters, otherwise the myocyte is unable to maintain steady state contractions.

The mechanisms underlying both activation and termination of Ca^{2+} release from the SR are controversial, however, the Ca^{2+}-induced Ca^{2+}-release mechanism is the most widely accepted. Several alternative mechanisms have been proposed as the main contributor in triggering Ca^{2+} release, such as Ca^{2+} influx through the T-type I_{Ca} ($I_{Ca,T}$) or through the Na^+/Ca^{2+} exchanger. However, I_{Ca} via these mechanisms is much less effective and slower than the flux through the L-type I_{Ca} ($I_{Ca,L}$) (Sipido *et al.*, 1997, 1998). Ca^{2+} release from smooth muscle SR and endoplasmic reticulum can also be triggered by inositol (1,4,5)-triphosphate (IP_3). High concentrations of IP_3 can induce Ca^{2+} release in cardiac myocytes via IP_3 receptors (Perez *et al.*, 1997), however, the rate and extent of Ca^{2+} release are much lower than that of the Ca^{2+}-induced Ca^{2+}-release mechanism (Lipp *et al.*, 2000).

1.1.4 Regulation of contractile force

The force of myocardial contractility is a variable, regulated quantity and can be increased by two processes, (i) when influenced by an intrinsic mechanism regulated by the degree of stretch of the myocytes during diastole or (ii) by nervous, hormonal or chemical stimulation.

1.1.4.1 The Frank-Starling mechanism

This intrinsic ability of the myocardium to adapt to changing preloads is known as the Frank-Starling mechanism. The degree of distension of the relaxed ventricle is determined by diastolic filling pressure. The pressure distending the RV, RV end-diastolic pressure (RVEDP), is almost equal to the central venous pressure (CVP) and similarly, the pulmonary venous pressure governs LA pressure and hence LV end-diastolic pressure (LVEDP). Initial fibre length at rest is proportional to the energy of the subsequent contraction, partly because it influences the actin-myosin overlap and to a greater extent because the sensitivity of the contractile machinery to free Ca^{2+} is length dependent (Kentish & Wrzosek, 1998; Fukuda *et al.*, 2001). Therefore, raising the filling pressure and diastolic volume increases contractile energy and stroke work. The most vital effect of the Frank-Starling mechanism is to balance the output of the right and left ventricles over several beats. The importance of which is observed when an imbalance between RV and LV outputs of a mere 1%, if sustained, can raise pulmonary blood volume sufficiently to induce pulmonary congestion and oedema.

In the intact circulation, venous return must equal cardiac output due to the closed nature of the circulation, therefore, venous return is directly dependent on cardiac output. In contrast, CVP is variable and can regulate stroke volume via the Frank-Starling mechanism as previously discussed. CVP is set by several factors, these include blood volume, gravity and peripheral venous tone. Dehydration or haemorrhage reduces blood volume and lowers CVP, while gravity can redistribute the blood to the lower limbs during long periods of standing (venous pooling). Sympathetic nerves that induce venoconstriction innervate the veins of the skin, kidney and splanchnic system. This controls the proportion of blood in the periphery and thereby influences CVP. Conversely, venodilation as a result of thermoregulation under hot conditions lowers CVP, reducing stroke volume.

1.1.4.2 The autonomic nervous system

Both divisions of the autonomic nervous system innervate the heart. The sympathetic division increases heart rate, the force of contraction and accelerates relaxation, while the parasympathetic division, via the vagus nerve, evokes bradycardia. Sympathetic fibres synapse with the electrical system and also directly innervate the atria and ventricles. The nerve endings release noradrenaline, which binds to β_1-adrenoceptors on the cardiac membrane. These receptors are coupled to a signal transduction pathway that activates the membrane bound enzyme adenylyl cyclase (AC) via a G-protein (G_s). The subsequent increase in cyclic 3',5'-adenosine monophosphate (cAMP) production activates protein kinase A (PKA), which phosphorylates proteins involved with excitation-contraction coupling such as phospholambam, L-type Ca^{2+} channels, ryanodine receptors and troponin I. The pacemaker cells are stimulated to increase the inward depolarising current (I_f), consequently raising the depolarising rate and the heart rate. β-adrenoceptor stimulation also induces positive inotropy as $[Ca^{2+}]_i$ is increased via the combination of increased inward I_{Ca} and the accumulation of a greater store of SR Ca^{2+}. The activity of phospholambam is increased by PKA, this protein is responsible for regulating the activity of the SR Ca^{2+}-ATPase (SERCA) pump and accelerates relaxation by increasing re-uptake of Ca^{2+} into the SR. Terminals of the vagus nerve synapse mainly on the SA and AV nodes, to a lesser extent on the atria and even less on the ventricles. The nerve endings release acetylcholine, which binds to muscarinic (M_2) receptors. These receptors are negatively coupled to AC, reducing cAMP accumulation, slowing the firing rate of the SA node via the right vagus and increasing the AV conduction delay via the left vagus to slow the heart.

1.1.4.3 Hormones, peptides and other factors

The endocardial endothelium plays an essential role in the heart with respect to cardiac metabolism, growth, contractile performance and rhythmicity (as reviewed by Shah *et al.*, 1996; Brutsaert, 2003). Endothelial cells can synthesise and release a variety of auto- and paracrine agents, such as NO, prostanoids, angiotensin II (Ang II), ET-1 and other substances, which can directly modulate the subjacent myocytes

(Discigil *et al.*, 1995; Mebazaa *et al.*, 1993a, 1993b; Dostal & Baker, 1999). These agents are often synergistic with others to produce novel biological outcomes, which would not necessarily be seen alone.

NO can be synthesised from L-arginine by three different nitric oxide synthase (NOS) isozymes. Endothelial constitutive (ecNOS, NOSIII) and neuronal (nNOS, NOSI) isoforms, are constitutively expressed in physiological conditions, while a third, inducible isoform (iNOS, NOSII), is biosynthesised only after stimulation by various stressors and cytokines. The ecNOS isoform is expressed in the coronary vascular endothelium, the cardiac endothelium and to a lesser extent in cardiomyocytes (Seki *et al.*, 1996; Andries *et al.*, 1998). Some studies have demonstrated that NO can induce a biphasic response, where low levels produce positive inotropic effects and high levels induce negative inotropic effects (Grocott-Mason *et al.*, 1994; Cotton *et al.*, 2001). However, studies have most consistently reported that NO produces isometric twitch relaxation *in vitro* and ventricular relaxation and enhanced diastolic compliance *in vivo*. These effects are mainly attributed to the activation of myocardial soluble guanylate cyclase to produce guanosine 3,5-cyclic monophosphate (cGMP), thereby evoking relaxation of the underlying myocardium (Paulus *et al.*, 1994; Mohan *et al.*, 1996; Flesch *et al.*, 1997), but some are cGMP independent. Cardiac responses to NO have been shown to be modified by other factors, these include the interaction between NO and the β_1-adrenergic and cholinergic pathways (Balligand, 1999; Gyurko *et al.*, 2000) or the interaction with the natriuretic peptides (Leskinen *et al.*, 1995).

Several prostanoids are synthesised and released from cardiac endothelial cells in response to various stimuli (Carter *et al.*, 1992; Mebazaa *et al.*, 1993a). Cyclooxygenase (COX) plays a key role in regulating prostaglandin (PG) synthesis, a constitutive (COX-1) isoform is expressed in all cardiac endothelial cells, while an inducible (COX-2) isoform is expressed during inflammatory states. Contractile responses to PGI_2 or PGE_2 have ranged from increased inotropy (Karazyn *et al.*, 1978; Katircioglu *et al* 1998) to no effect (Couttenye *et al.*, 1985) and negative inotropy (Schror *et al.*, 1992). Studies have attributed the inconsistent responses to

the interaction of PG with NO as there is continuous crosstalk between the NOS and COX pathways (Gerritsen, 1996), therefore, the inotropic actions result from the ratio between cAMP-cGMP.

Ang II is released via the activity of angiotensin converting enzyme (ACE) and also via an ACE-independent pathway. Cardiac ACE is predominantly expressed in endothelial cells but can also be found in cardiomyocytes and fibroblasts (Dostal & Baker, 1999). The effects of Ang II on cardiac contractile performance are thought to result from locally produced rather than circulating Ang II (Dzau, 1988). Ang II generally exerts a positive inotropic effect partly by enhancing the Ca^{2+} plateau current and also by acting at sympathetic nerve endings to facilitate the release of noradrenaline, to increase the rate of rise of the contractile phase and delay relaxation (Baker & Singer, 1988; Meulemans *et al.*, 1990). However, there have also been inconsistent responses reported and these reflect the many interactions that modify the cardiac synthesis, release and activity of Ang II. Studies have demonstrated interactions with bradykinin, the NO and PGI_2 pathways as well as ET-1 (Ritchie *et al.*, 1998a, 1998b; Kusaka *et al.*, 2000). ET-1 is also synthesised and released in the heart and its effects on cardiac contractility and venous tone are discussed in section 1.2.6.

In the heart, local extracellular electrolyte disturbances such as hyperkalaemia can significantly alter cardiac function. In addition, other situations have negative inotropic responses. Ischaemia is associated with hypoxia and intracellular acidosis, both of which can depress contractility. Hypoxia results in an increased concentration of inorganic phosphates, which interfere with Ca^{2+} binding and also raises the intracellular H^+ concentration. Acidosis reduces the sensitivity of the myofibrils to Ca^{2+}, thereby reducing the force of contraction. Endogenous agents such as the circulating catecholamines, adrenaline and noradrenaline, can stimulate cardiac β-adrenoceptors, inducing inotropic and chronotropic responses (as discussed above). Other factors that alter the contractile state of the heart include caffeine and drugs like digoxin, which act on the SR membrane to increase the frequency of opening of the Ca^{2+}-release channels. In addition, caffeine inhibits

phosphodiesterase, the enzyme that breaks down the second messenger cAMP, thereby elevating cAMP and inducing actions similar to β_1-adrenergic stimulation.

1.2 The Endothelin system

1.2.1 Endothelin-1

The vascular endothelium is recognised as an important source of vasoactive factors. In both health and disease, it modulates vascular tone through a variety of substances that are locally synthesised and released. ET-1 is a potent vasoconstrictor peptide produced by the vascular endothelial cells (Yanagisawa *et al.*, 1988a). It is one of a family of peptides which are characterised by their unique shape, each are comprised of 21 amino acid residues, including two disulphide bonds linking cysteine amino acids between residues 1 to 15 and 3 to 11 and six conserved amino acid residues at the carboxy terminal (*Figure 1.2*). Three isoforms of ET have been identified, of which ET-1 is predominant in the mammalian cardiovascular system. Localisation of the peptide suggests that ET-2 may possibly function as a mediator in the human kidney (Karet & Davenport, 1996), while ET-3 may act as a mediator in the kidney, gut and nervous system. Sarafotoxins (SRTXs), peptides isolated from the venom of the Israeli burrowing asp, *Actactaspis engaddensis* (Kloog *et al.*, 1988), share close structural similarity to ET isopeptides. Both ETs and SRTXs act through common receptors to evoke a multitude of effects. SRTX S6c (*Figure 1.2*) in particular, has been used as a tool for characterising the actions of ET-1 at its receptors (Sokolovsky, 1994).

1.2.2 Biosynthesis of ET-1

In the human genome, distinct genes encoding the precursors for ET-1, -2 and -3 are found on chromosomes 6, 1 and 20 respectively (Bloch *et al.*, 1989a, 1991, 1989b). All three ETs are synthesised through a two-stage process from their respective precursor peptides. In the case of human ET-1 (*Figure 1.3*), ET-1 mRNA is translated in the nucleus to preproET-1, a 212 amino acid polypeptide and a short terminal signal sequence (amino acids 1-17) is removed. The resulting propeptide is then secreted into the cytoplasm where it is cleaved at two specific sites containing pairs of dibasic amino acids by furin-like endopeptidases. In the first proteolytic step, the polypeptide cleaves between Lys^{52}-Arg^{53} and Arg^{90}-Arg^{91}, releasing an intermediate 'big ET-1' which has 38 amino acids and is biologically inactive.

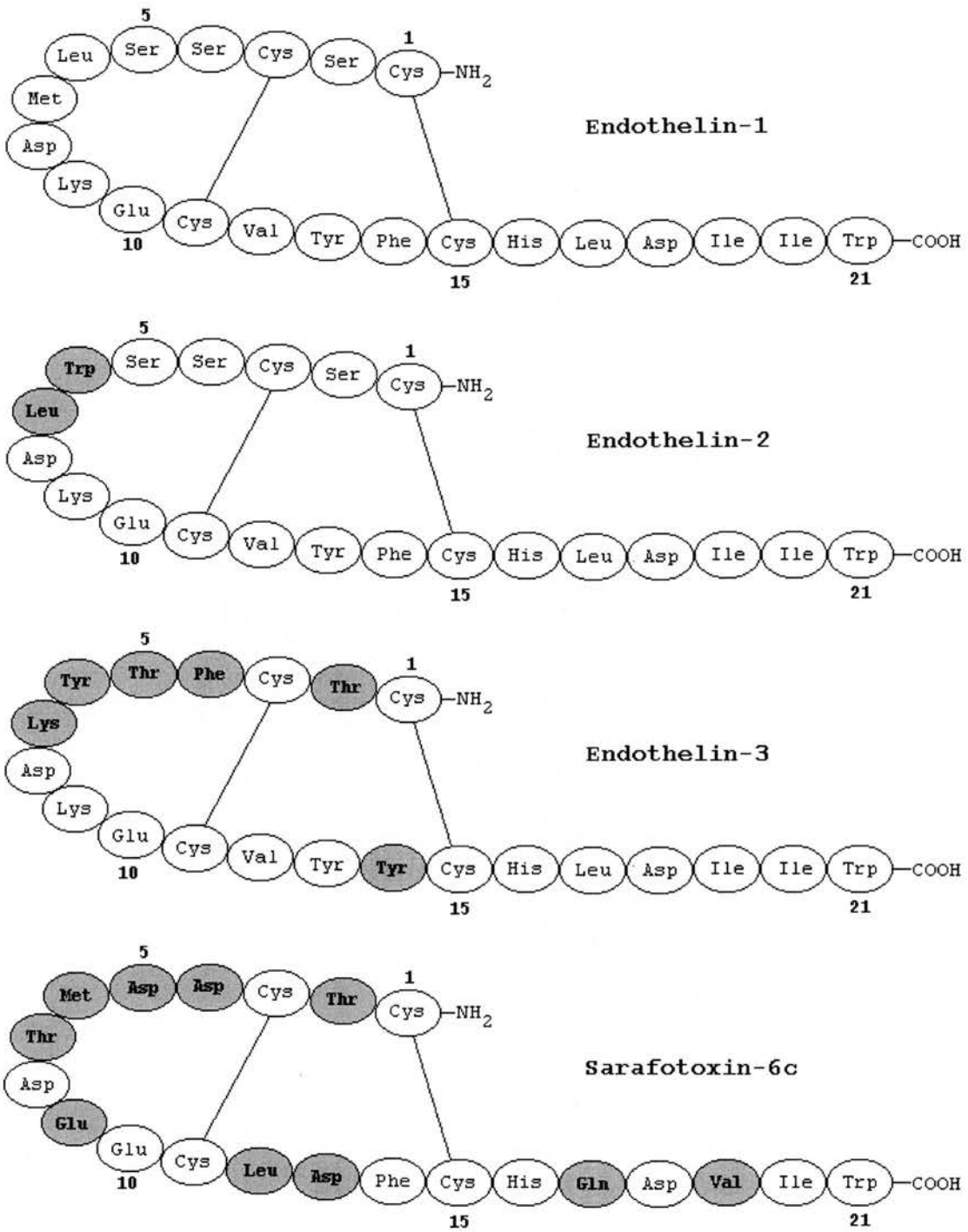


Figure 1.2 The unique structure of the endothelin isopeptides and sarafotoxin S6c. The filled circles indicate that the amino acid differs from the ET-1 sequence.

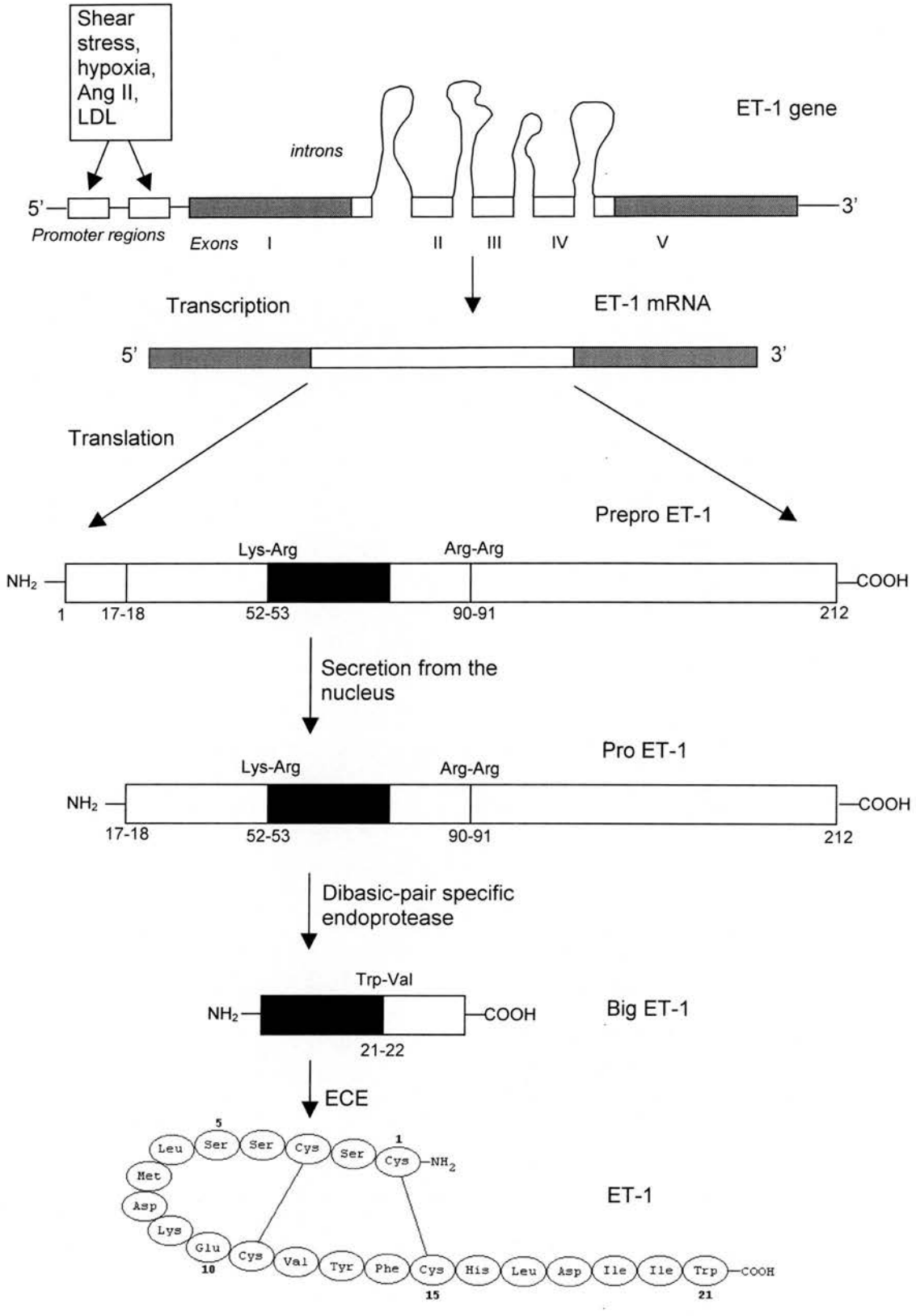


Figure 1.3. Biosynthesis of ET-1 and the signalling pathways involved in gene regulation. Modified from Gray & Webb (1996).

Subsequently, mature ET-1 is formed by cleavage at the c-terminal between Trp^{21}-Val^{22}, catalysed by a specific endothelin converting enzyme (ECE) (Yanagisawa *et al.*, 1988b). Big ET-1 is several orders of magnitude less active than ET-1 (<10-fold) and inhibition of ECE by the neutral metalloendopeptidase inhibitor phosphoramidon, almost completely inhibits the pressor action of big ET-1, indicating that conversion of big ET-1 to ET-1 is essential for biological activity (Haynes & Webb, 1994).

Takahashi *et al.* (1993) were the first to purify and characterise ECE from rat lung. Subsequently, cloning and expression of ECE-1 was demonstrated in rat lung vascular endothelial cells (Shimada *et al.*, 1994) and cultured human and bovine heart endothelial cells (Schmidt *et al.*, 1994), with ECE-1 being identified as a membrane bound neutral metalloprotease. ECE-1 is a type II integral membrane protein composed of 754 or of 758 amino acids, it has a neutral pH optimum and has been shown to be localised on the cell surface as well as intracellularly in a variety of cells including endothelial cells (Barnes *et al.*, 1996). The enzyme has a short N-terminal domain, a single transmembrane segment and a large C-terminal domain, which contains the active site of the enzyme. Three ECE-1 isoforms have been identified in the human (ECE-1a, ECE-1b and ECE-1c), they are derived from a single gene and differ only in a variation in their N-terminal region through the use of alternative promoters. They cleave big ET-1 with similar efficiency but differ with respect to their subcellular localisation (Schweizer *et al.*, 1997). In addition, the existence of a fourth human ECE-1 (ECE-1d) was demonstrated in cultured cells and shown to have similar biochemical properties to the other isoforms (Valdenaire *et al.*, 1999). ECE-1a is most strongly expressed at the cell surface, while ECE-1b is almost exclusively intracellular. ECE-1c is the major isoform in most human tissues and is moderately expressed on the plasma membrane while ECE-1d is localised moderately on both the cell surface and intracellular structures (Schweizer *et al.*, 1997; Valdenaire *et al.*, 1999).

ECE-2, a second ECE subtype was cloned by Emoto & Yanagisawa (1995). It is homologous to ECE-1 but differs principally in sensitivity to phosphoramidon and in

pH optimum, requiring acidic conditions (pH 5.5-5.7). ECE-2 cDNA is located in diverse cell types but seems most abundantly expressed in neural tissues (Emoto & Yanagisawa, 1995). Both show preference for big ET-1 over ET-2 and ET-3 however, targeted disruption of the ECE-1 gene in mice suggests that ECE-1 is the predominant subtype responsible for the conversion of big ET-1 to ET-1 (Yanagisawa *et al.*, 1998).

1.2.3 Regulation of the ET-1 gene

Regulation of endothelin synthesis is thought to occur at the level of transcription, with de novo production and release occurring in response to stimulation. Upregulation of ET-1 mRNA has been shown in a variety of cells in response to vasoactive hormones, inflammatory mediators and physicochemical factors such as shear stress and hypoxia (*Table 1.1*). These factors activate a protein kinase C (PKC) / phospholipase C (PLC) mediated increase of gene transcription (Lee *et al.*, 1991). In contrast, inhibition of ET-1 generation occurs via cGMP in response to nitric oxide, natriuretic peptides and prostacyclin (*Table 1.1*). Promotor regions located at the 5' gene flanking sequence of the ET precursor serve to control the rate of transcription by mediating the effects of these diverse stimuli (Benatti *et al.*, 1993).

Release of ET-1 from the endothelial cells of blood vessels or endocardial endothelial cells of the heart, is predominantly abluminal, towards the underlying vascular smooth muscle cells (SMCs) or cardiomyocytes. Local ET-1 concentration within the vascular wall is ≥100-fold higher than that of plasma levels, stemming in part from the fact that 80% of the ET-1 is secreted on the basal side of endothelial cells (Wagner *et al.*, 1992). As a consequence, big ET-1 and ET-1 are present in plasma at low concentration (~1pM), while circulating concentrations of ET-2 and ET-3 are even lower (Battistini *et al.*, 1993). Therefore, ET peptides are generally thought to act as autocrine and paracrine factors, rather than circulating hormones. (Yoshimoto *et al.*, 1991). Although ET-1 is identified within endothelial cells, it remains unclear whether intracellularly stored peptide represents a pool available for rapid release.

Initial localisation studies reported that the processing of big ET-1 to mature ET-1 was attributed to the activity of a converting enzyme that was located on the plasma membrane of human (Waxman *et al.*, 1994), bovine (Corder *et al.*, 1995) and rat (Takahashi *et al.*, 1995) cultured vascular endothelial cells, acting mainly in a post-secretory manner. In contrast, other studies have suggested that ECE is either primarily expressed or has predominant activity within intracellular compartments (Gui *et al.*, 1993; Xu *et al.*, 1994). Localisation of intracellular ECE has been demonstrated within the Golgi apparatus and discrete secretory vesicles, implicating conversion of endogenous big ET-1 during its transit through the cell cytosol (Corder & Barker, 1999). This is consistent with the localisation of both ET-like and ECE-like immunoreactivity within storage granules, 'Weibel Palade bodies', in human coronary artery endothelium (Russell *et al.*, 1997, 1998). Together with functional experiments showing rapid, stimulated release of ET-1 *in vivo* and *in vitro* (Fyhrquist *et al.*, 1990; Macarthur *et al.*, 1994), these observations suggest that a regulated pathway may also be important in the processing of big ET-1. Thus, two distinct secretory pathways play a role in the secretion of ET-1: a constitutive pathway, controlled at the level of protein synthesis, involving basal release of ET-1, and a regulated pathway of release involving the stimulated mobilisation and degranulation of endothelial cell-specific storage granules.

It has been observed that many endothelial cell stimulants such as thrombin (Boulanger & Lüscher, 1990) cause a delayed (i.e. hours), but not immediate (i.e. minutes) elevation in the production of ET-1, which appears to depend upon an increased synthesis of the peptide. In contrast, some stimuli, such as cold stress (Fyhrquist *et al.*, 1990) can cause an immediate increase in the circulating levels of endothelin. Stretching of endothelial cells has also been shown to produce an elevation in ET-1 production. Macarthur *et al.* (1994) demonstrated a rapid (≤20 minutes) and a prolonged (≥360 minutes) increase of ET-1 accumulation in bovine aortic endothelial cells after stretching, indicating that both *de novo* synthesis and release from intracellular stores is involved. Similarly, Hishikawa *et al.* (1995) demonstrated a pressure induced ET-1 release from cultured human umbilical vein endothelial cells (HUVEC), mediated partially through activation of PLC and PKC.

Furthermore, Kuchan and Frangos (1993) observed that the level of shear stress applied to the endothelial cells affected the ET-1 release. While exposure to a low level of shear stress (1.8 dyne/cm^2) resulted in a sustained increase in cumulative ET-1 release via PKC activation, higher levels of stress (≥6 dyne/cm^2) progressively inhibited PKC mediated ET-1 mRNA transcription in a dose dependent manner. As endothelial cells are continuously exposed to blood flow and the resultant mechanical forces, it implies an important role of ET-1 in the acute and chronic control of local blood flow. In addition, mechanical stretching has been shown to stimulate the secretion and production of ET-1 in cultured rat ventricular cardiomyocytes. This is associated with an increase of ET-1 mRNA levels, as evidenced by Northern blot analysis (Yamazaki *et al.*, 1996).

Table 1.1. Factors influencing the gene transcription of ET-1

Factor	Effect	Cellular signal	Reference
Angiotensin II	Increase	PLC/PKC	Emori *et al.*, 1991
Vasopressin	Increase	PLC/PKC	Emori *et al.*, 1991
Insulin	Increase	PLC/PKC	Oliver *et al.*, 1991
TGF-β	Increase		Kurihara *et al.*, 1989
Cyclosporin	Increase	TGF-β	Takeda *et al.*, 1993
Hypoxia	Increase		Elton *et al.*, 1992
Shear stress (low)	Increase	PKC	Kuchan & Frangos *et al.*, 1993
Thrombin	Increase	PLC/PKC	Emori *et al.*, 1992
Oxidised LDL	Increase	PLC/PKC	Boulanger *et al.*, 1992
PGI_2/PGE_2	Decrease	cGMP	Prins *et al.*, 1994
Nitric oxide	Decrease		Boulanger & Lüscher 1990
Natriuretic peptides	Decrease	cGMP	Kohno *et al.*, 1992
Heparin	Decrease	NO/cGMP	Yokokawa *et al.*, 1993
Shear stress (high)	Decrease	NO/cGMP	Kuchan & Frangos *et al.*, 1993

PLC, phospolipase C; PKC, protein kinase C; TGF-β, transforming growth factor-β; PGI_2, prostacyclin; PGE_2, prostaglandin E_2; cGMP, cyclic GMP; NO, nitric oxide.

1.2.4 Sites of ET-1 generation

The gene for ET-1 has been detected in a wide variety of tissues. It is predominantly expressed in vascular endothelial cells, as initially demonstrated by Yanagisawa *et*

al. (1988a), in both cultured endothelial cells and in porcine aortic intima *in situ*. ET-1 is also released by human endothelial cells in culture (Clozel & Fischli, 1989) and immunoreactive ET-1 has been detected in the endothelial layer of human blood vessels of varying origin (Howard *et al.*, 1992; Tokunaga *et al.*, 1992). Cultured vascular SMCs from human (Resink *et al.*, 1990; Yu & Davenport, 1995), rat and rabbit (Kanse *et al.*, 1991) have also demonstrated mRNA expression of ET and peptide production. Studies have identified mRNA expression and immunoreactive ET-1 in endothelial cells from rat heart (Giaid *et al.*, 1991) and cultured rat cardiomyocytes (Suzuki *et al.*, 1993; Ito *et al.*, 1993). Previous studies in our own laboratory have confirmed this, demonstrating expression of ET-1 mRNA and *in situ* immunoreactive ET-1 localised in cardiomyocytes, myoendothelial cells and some vascular endothelial cells of the rat heart (Sherry, 2000). In addition, cultured ovine endocardial endothelial cells (Mebazaa *et al.*, 1993b), cultured human right ventricle endothelial cells (Plumpton *et al.*, 1996) and porcine cardiomyocytes in culture (Tønnessen *et al.*, 1995) express ET-1 mRNA. Immunostaining for ET-1 was localised in vascular and endocardial endothelial cells as well as cardiomyocytes of explanted human hearts (Giaid *et al.*, 1995), while in contrast, Davenport *et al.* (1998) did not detect ET-1 in human cardiomyocytes, suggesting instead that in humans, release of ET-1 from endocardial endothelial cells modulates myocardial function in a paracrine manner.

Evidence suggests that cultured rat epicardial mesothelial cells are able to express, synthesise and release ET-1 (Eid *et al.*, 1994; Kuwahara & Kuwahara, 1998). Additionally, high concentrations of the peptide have been reported in the pericardial fluid of humans and dogs (Horkay *et al.*, 1998; Szokodi *et al.*, 1998). Furthermore, strong immunoreactivity for ET-1 was observed in the endothelium of human epicardial coronary arteries and veins (Opgaard *et al.*, 1994), while both cultured endothelial cells and SMCs from the coronary artery have also been shown to release ET-1 (Ohlstein *et al.*, 1998; Haug *et al.*, 1998). *In vitro* studies have also demonstrated that neonatal rat cardiac fibroblasts can synthesise ET-1 when stimulated by other cardiac vasoactive factors (King *et al.*, 1996; Gray *et al.*, 1998).

1.2.5 ET receptors

Not long after the discovery of ET-1, two high affinity receptor subtypes were identified and cloned (Arai *et al.*, 1990; Sakurai *et al.*, 1990; Lin *et al.*, 1991). These specific binding sites for ET-1 were classified according to their relative affinities for the ET isopeptides. The ET_A receptor has subnanomolar affinities for the ET-1 and ET-2 peptides and a 70-100 fold lower affinity for ET-3 (ET-1>ET-2>>ET-3). The ET_B subtype has equal subnanomolar affinities for all three ET peptides and SRTX S6c (ET-1=ET-2=ET-3=SRTX S6c) (Sakurai *et al.*, 1990).

The human ET_A receptor gene has 8 exons and 7 introns, is assigned to chromosome 4 and encodes for a 427 amino acid polypeptide (Hosada *et al.*, 1992). The gene for the human ET_B subtype is assigned to chromosome 13, has 7 exons and 6 introns and encodes a 442 amino acid polypeptide (Arai *et al.*, 1993). Both predict classic heptahelical G-protein coupled receptors. Sequence homology of 55-64% has been found between the two, depending on the tissue studied (Arai *et al.*, 1993). The cytoplasmic loops of the receptors are highly conserved, but the N-terminal and other extracellular domains differ in both length and amino acid sequence (Ogawa *et al.*, 1991; Elshourbagy *et al.*, 1993). Specific regions along the sequences have been identified as essential for receptor activity, as well as signalling domains located within the c-terminal region (Sakamoto *et al.*, 1993). Of the seven transmembrane domains, I, II, III and VII are involved in ligand receptor binding, while IV, V and VI domains determine isopeptide selectivity (Sakamoto *et al.*, 1993). The third cytoplasmic domain of the ET_A receptor is very short (~30-50 residues) and is implicated in coupling with the G-protein (Adachi *et al.*, 1993).

Based on pharmacological studies, the ET receptors have been further classified. Sudjarwo *et al.* (1994) proposed subclassification of the ET_A receptor by its susceptibility to BQ123, with a sensitive ET_{A1} and resistant ET_{A2} subtype. Two types of ET_B receptor were also proposed, ET_{B1} and ET_{B2} were subclassified by their existence on different effector cells (endothelial cells or SMCs) (Masaki *et al.*, 1994; Douglas *et al.*, 1994). A third putative ET_C receptor with selective affinity for ET-3 was proposed in *Xenopus laevis* dermal melanophores (Karne *et al.*, 1993), and later

in rabbit saphenous vein (Douglas *et al.*, 1995), but has not been detected in human tissue. However, it is acknowledged that there is no genetic basis for the existence of these proposed subtypes, as only ET_A and ET_B receptor genes have been identified.

The development of ET receptor agonists and antagonists (*Table 1.2*) has enabled the selective study of the ET receptor subtypes in relation to the physiological and pathophysiological function of ET-1 in the cardiovascular system. However, to date, no selective ET_A receptor agonist has been discovered. In addition, gene knockout (KO) mice were developed to help characterise the function of the ET system components. However, many of the phenotypes were lethal, indicating the importance of the ET system in development. ET-1 and ET_A receptor gene deficient mice die shortly after birth due to respiratory failure and exhibit severe craniofacial malformations, aortic and ventricular septal defects also exist in these mice (Kurihara *et al.*, 1994; Clouthier *et al.*, 1998; Yanagisawa *et al.*, 1998). Most ECE-1 KO mice die earlier in utero but still exhibit craniofacial abnormalities (Yanagisawa *et al.*, 1998). Meanwhile, although ET-3 and ET_B gene deficient mice are viable at birth, they die 2-4 weeks after from intestinal dysfunction due to aganglionic megacolon, resulting from the absence of the enteric neurons (Baynash *et al.*, 1994; Hosada *et al.*, 1994). Interestingly, mutations of both ET-3 and ET_B have also been reported in patients of both sporadic and familial Hirschsprung's disease with or without pigmentatory abnormalities (Puffenberger *et al.*, 1994; Edery *et al.*, 1996), highlighting the importance of ET-3 and ET_B mediated signalling in development of enteric neurons and melanocytes in humans.

Table 1.2. ET receptor agonists and antagonists

Category	ET_A	ET_B	Reference
ET potency order	ET-1>ET-2>>ET-3	ET-1=ET-2=ET-3	Sakurai *et al.*, 1990
Selective agonist	none	SRTX S6c	Williams *et al.*, 1991
Selective antagonist (peptide)	BQ123 FR139317 LU135252 PD156707		Ihara *et al.*, 1991 Aramori *et al.*, 1993
		BQ788 IRL2500	Ishikawa *et al.*, 1994 Balwierczak *et al.*, 1995
Selective antagonist (non peptide)	BMS182874	Ro 468443	Stein *et al.*, 1994 Brändli *et al.*, 1996
Non selective antagonist	TAK-044 (peptide) Bosentan (Ro 47-0203) (non peptide) SB209670 (non peptide)		Kikuchi *et al.*, 1994 Clozel *et al.*, 1994 Ohlstein *et al.*, 1994

1.2.6 ET receptor localisation, function and signalling

1.2.6.1 Vascular ET receptors

ET_A and ET_B receptors have different vascular distributions. *In situ* hybridisation and Northern blotting techniques have revealed that in blood vessels, the ET_A receptor is predominately expressed in SMCs of bovine lung (Arai *et al.*, 1990), bronchial SMCs, renal arterioles, brain blood vessels and the aorta of the rat (Hori *et al.*, 1992; Sakurai *et al.*, 1990; Maxwell *et al.*, 1998) and human cerebral arteries, pulmonary arteries and the aorta (Nilsson *et al.*, 1997; Davenport *et al.*, 1993). Meanwhile, various techniques have demonstrated that ET_B receptors exist predominantly on endothelial cells but they are also detected on vascular SMCs of similar vessels including hepatic tissue (Leivas *et al.*, 1998), aorta and pulmonary arteries (Davenport *et al.*, 1993), human cerebral arteries (Nilsson *et al.*, 1997) and glial cells of many brain regions (Hori *et al.*, 1992). However, in contrast, ET_A is absent from human endothelial cells (Hosada *et al.*, 1991). The ratio of the two receptors on SMCs varies depending on the vascular bed, with veins in general having lower ET_A: ET_B ratios than arteries (Lipa *et al.*, 1999).

The role of endogenous ET-1 has been investigated in many animal and human studies. Evidence shows that ET-1 has a role in maintenance of basal vascular tone and blood pressure (Haynes & Webb *et al.*, 1994). It has also been shown that both receptors are involved in mediating the effects of ET-1 (Clozel *et al.*, 1992) and the rapid development of potent and selective ET receptor agonists and antagonists has allowed the functional role of ET receptors to be more clearly defined (*Table 1.2*).

Intravenous bolus administration of ET-1 is known to cause a short-lived decrease (up to a few minutes) in vascular resistance, followed by a marked pressor effect ($\geq$ 1 hour) in both humans and animals (Yanagisawa *et al.*, 1988a; Vierhapper *et al.*, 1990; Pernow *et al.*, 1996). The initial increase in perfusion is caused via NO and PGI_2 release by ET_B stimulated endothelial cells as studies have shown that ET_B antagonists (such as BQ788; Douglas *et al.*, 1994) and inhibition of both PGI_2 and NO synthesis (Filep *et al.*, 1993; Berti *et al.*, 1993) abolish the dilator response both *in vivo* and *in vitro*. Meanwhile, ET_A receptors on SMCs primarily mediate the vasoconstriction as infusion of selective ET_A receptor antagonist, BQ123, leads to an increase in local blood flow in human forearm vasculature studies (Haynes & Webb, 1994) and greatly attenuates the pressor response in rats (Bird *et al.*, 1993). However, BQ123 does not completely abolish the pressor response (McMurdo *et al.*, 1993) and ET_B selective agonists have been shown to evoke constriction *in vitro* (Shetty *et al.*, 1993; Gray *et al.*, 1994) and pressor responses *in vivo* (Clozel *et al.*, 1992). Therefore, taken together, these observations are consistent with the view that endothelial cell ET_B receptors mediate the dilator response, SMC ET_A receptors predominately mediated the constrictor response and that ET_B receptors are also present on vascular SMCs to stimulate constriction (*Figure 1.4*).

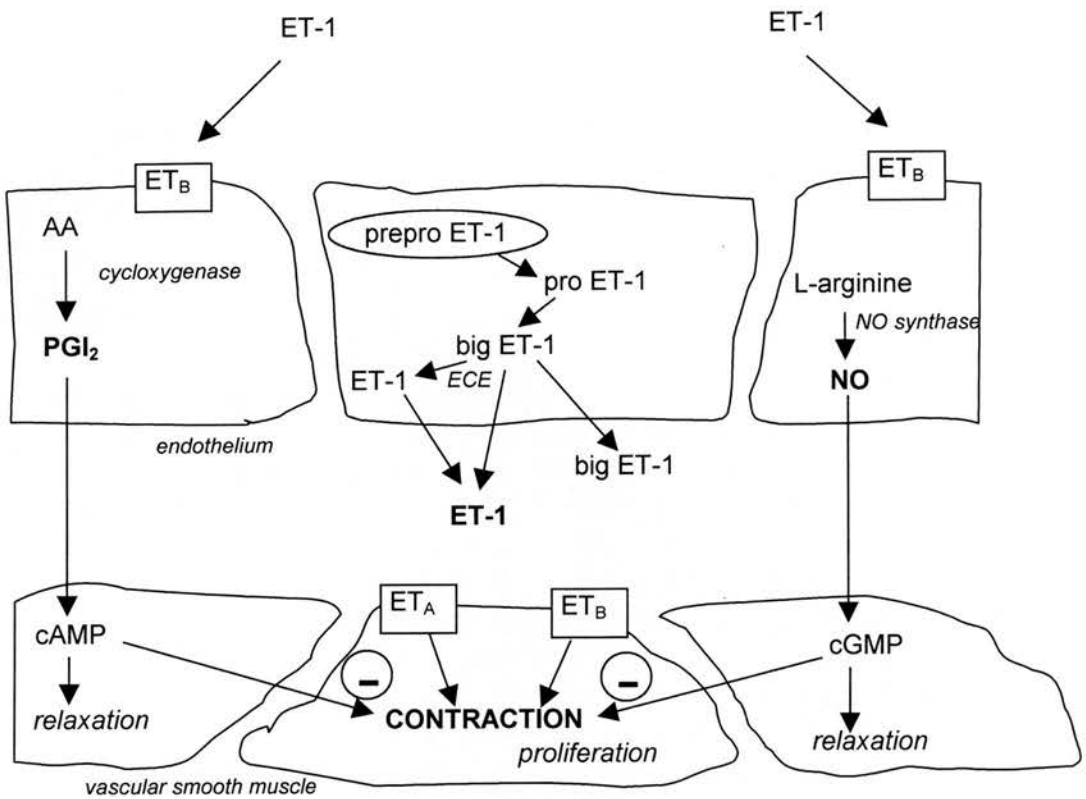


Figure 1.4. Endothelium derived factors that regulate vascular smooth muscle tone. AA, arachidonic acid; ECE, endothelin converting enzyme; NO, nitric oxide; PGI_2, prostacyclin. Modified from Gray & Webb, (1996).

ET-1 induced vascular contraction is rapid and utilises various signalling mechanisms. ET receptors on SMCs are G-protein-coupled, resulting in activation of PLC, and generation of two second messengers, IP_3 and 1,2-diacylglycerol (DAG) (Griendling *et al.*, 1989), which respectively stimulate Ca^{2+} release and protein kinase C activation (Simpson & Ashley, 1989; Lonchampt *et al.*, 1991). ET_B receptors on endothelial cells mediate relaxation of vascular SMCs via release of NO and PGI_2. The G-protein-coupled receptor mobilises Ca^{2+} via PLC (as described above), generating a Ca^{2+}/Calmodulin complex, which in turn activates NOS, the enzyme which is central to NO biosynthesis. Diffusion of NO occurs freely, enabling it to leave the endothelial cell and cross over to the SMC, where depending on its concentration, NO will induce an increase of cGMP (Ignarro, 1989). Elevated levels of cGMP act on myosin light chain kinase, PKG, phosphodiesterases and ion channels, resulting in reduced $[Ca^{2+}]_i$ (Moncada & Higgs, 1991). A PKG-dependent mediated reduction of myofilament responsiveness to Ca^{2+} also adds to the negative contractile effect. Conversely, a positive contractile response is induced by an increase of cAMP, induced by low level NO stimulation of a novel cGMP-independent activation of AC (Kojda *et al.*, 1996).

Increased concentration of circulating ET-1 has been observed when mixed ET_A/ET_B and selective ET_B antagonists but not selective ET_A antagonists are infused in rat, rabbit and human (Fukuroda *et al.*, 1994; Gratton *et al.*, 1997; Strachan *et al.*, 1999), suggesting a role for ET_B receptors in the clearance of ET-1. The lungs are the organ containing the highest density of ET_B receptors, with close to 50% of the endothelium of the entire vascular tree. Considering the high blood volume in that organ at any time, the pulmonary endothelium could act as a very efficient clearing mechanism (Dupuis *et al.*, 1996). Extraction of ET-1 follows binding to cell surface receptors which are then internalised, allowing degradation to be carried out within the cell (Gandhi *et al.*, 1993). The ligand-receptor complex is thought to be sequestered into lysosomes where the acidic environment promotes ligand dissociation (Hirata *et al.*, 1988). Other organs such as kidneys and liver are also reportedly involved in the clearance of circulating ET-1 by ET_B receptors (de Nucci *et al.*, 1988; Dupuis *et al.*, 1999). Conversely, ET_A receptors are not thought to be

involved in clearance of plasma ET-1 as internalised ET_A receptors are recycled back to the cell surface (Bremnes *et al.*, 2000).

1.2.6.2 ET receptors in the kidney

The ET system is known to play an important role in the regulation of vascular tone and blood pressure. Studies have also indicated a role for ET-1 as a key natriuretic hormone (as reviewed by Kotelevtsev & Webb, 2001). The kidney is uniquely influenced by ETs and a wide variety of renal cell types express mRNA for the receptors and the precursors of ET-1 and ECEs (Terada *et al.*, 1992; Kohan, 1997). The ET system controls renal blood flow, reabsorption of water and sodium and acid-base balance (King *et al.*, 1989; Bloom *et al.*, 1993). ET-1 administration into the renal artery of an anaesthetised rabbit results in reduced renal blood flow, cortical perfusion, glomerular filtration rate, urinary flow and sodium excretion (Evans *et al.*, 1998). Systemic infusion of ET-1 in humans also causes renal vasoconstriction and decreases renal sodium excretion (Sørensen *et al.*, 1994). In contrast, administration of big ET-1 into the femoral artery of rats produces a significant increase in sodium and water excretion that cannot be blocked by BQ123, the ET_A receptor antagonist. This is due to the activation of ET_B receptors deep in the medulla, causing natriuresis and diuresis (Pollock & Opgenorth, 1994), indicating that ET-1 has different functions in the renal tubule than in the vasculature. The role of ETs in sodium excretion and acid-base status has been further explored in rats and mice genetically lacking ET_B receptors (Gariepy *et al.*, 1998; Ohuchi *et al.*, 1999). These animals exhibit significant hypertension and data suggests that ET_B likely acts within the kidney, not in the vessel wall, to regulate pressure. *In vivo*, it is suggested that ET_B receptors in the collecting duct may tonically inhibit sodium reabsorption via luminal epithelial sodium channels, the final regulator of sodium balance, as the sodium channel blocker amiloride, restores normal mean arterial blood pressure (MAP) in the salt-loaded ET_B deficient animals (Gariepy *et al.*, 2000).

1.2.6.3 Cardiac ET receptors

Autoradiography has been used to demonstrate binding of [^{125}I] ET-1 to both ET_A and ET_B receptors in human coronary arteries and veins. The ET_A receptor subtype is predominant in coronary artery SMCs (Bacon & Davenport, 1996), while, ET_B receptor binding is weak at the proximal regions but increases in density towards distal regions of the vessel (Davenport *et al.*, 1994). Meanwhile, ET_B expression is exclusively on the coronary endothelium (Davenport *et al.*, 1993; Opgaard *et al.*, 1996). In addition, these studies indicated that ET-1 induced contractile response was mediated only by ET_A receptors, where as ET_B receptors mediate relaxation. In animal studies, quite a different receptor profile has been reported. Antagonist studies have identified that contraction of pig coronary artery is mediated by both receptors (Schoeffter & Randriantsoa, 1993) and the canine coronary circulation also contains a significant population of ET_B receptors that can mediate both vasodilation and vasoconstriction (Teerlink *et al.* 1994).

In the myocardium, ET_A and ET_B receptors have a similar distribution, with mRNA of both found on cardiomyocytes of many species, including rat (Sargent *et al.*, 1994; Sakai *et al.*, 1996b), rabbit (Spiers *et al.*, 1996) and human (Molenaar *et al.*, 1993; Davenport *et al.*, 1993). Binding studies have demonstrated that both subtypes are located in atrial and ventricular myocardium, although their distribution is not uniform. Significant regional differences were shown in the human heart by Molenaar *et al.* (1993), indicating ET_A receptor density to be greatest in isolated right atrial myocytes (91%), while in myocytes of the LV free wall, the ET_A subtype contributes approximately 57-67% of the total population. However, the ratio of the receptors differs depending on the tissue and species, with the ET_A receptor subtype representing 90% of the endothelin receptors on rat right ventricular cardiomyocytes (Sakai *et al.*, 1996a; Fareh *et al.*, 1996). In contrast, *in situ* studies in our own laboratory have yielded a 80:20 distribution of ET_A:ET_B in LV (Sherry, 2000).

Both receptors are also found within the AV conducting system. Localisation using autoradiography in the human heart has shown a higher proportion of ET_B receptors within the AV node, the penetrating and branching bundles of His, than in the

surrounding atrial and ventricular myocardium (Molenaar *et al.*, 1993). Fibroblasts display an equal distribution of the receptor subtypes in rat (Fareh *et al.*, 1996), but not human (Modesti *et al.*, 1999), where *in situ* hybridisation studies have demonstrated that ET_B receptor mRNA was almost exclusively expressed in fibroblasts and endothelial cells of the human LV. In addition, functional ET_B receptors have also been identified on rat pericardial mesothelial cells (Kuwahara & Kuwahara, 1998).

Like ET-1, expression of ET receptors is also influenced by various circulating factors. Studies have shown that in cultured rat vascular SMCs, ET_A is upregulated by insulin and NO (Frank *et al.*, 1993; Redmond *et al.*, 1996). While expression of ET_B receptor mRNA was shown to be upregulated in cultured rat cardiomyocytes by Ang II (Kanno *et al.*, 1993) and in human HUVECs by cytokines and growth factors (Smith *et al.*, 1998). In addition, ET-1 itself has been shown to alter receptor number (Hirata *et al.*, 1988). Increasing evidence also shows that receptor expression is modified in the diseased state (Batra *et al.*, 1993; Sakai *et al.*, 1996a; Gray *et al.*, 2000) (to be discussed later in section 1.3).

The role of the ET system in contractile function of the heart is still not clearly defined. Clinical studies have shown that intracardiac infusion of the ET_A selective antagonist BQ123 in healthy volunteers causes a significant reduction in LV dP/dt, a measurement of contractility, with no effect on relaxation and an absence of systemic effects such as heart rate, aortic pressure or pulmonary artery pressure (MacCarthy *et al.*, 2000). In contrast, systemic infusion of the mixed antagonist TAK-044 in healthy subjects increased cardiac index (Haynes *et al.*, 1996). However, this was attributed to the associated decrease in systemic vascular resistance, suggesting that a large part of the effect is mediated in the resistance vessels. Meanwhile, systemic administration of BQ788, the selective ET_B receptor antagonist, caused a significant increase in MAP, increased total peripheral vascular resistance and reduced heart rate, cardiac index and renal blood flow (Strachan *et al.*, 1999), due in part to reduced clearance of ET-1 from the circulation. Furthermore the pressor effect observed in anaesthetised dogs after bolus administration of ET-1 is associated with

extreme peripheral vasoconstriction, a reduced cardiac output, bradycardia and consequent activation of baroceptor reflexes (Given *et al.*, 1989). Heart rate was not influenced by ET-1 in anaesthetised rats but cardiac output was reduced due to the decrease of stroke volume (Beyer *et al.*, 1994). Consequently, it has proved difficult to distinguish the functional characteristics of ET-1 *in vivo* due to direct and indirect effects.

Difficulties in obtaining positive inotropic responses to ET-1 have also been observed in studies using isolated hearts from rats (Neubauer *et al.*, 1990), or rabbit (Karawatowska & Wennmalm, 1990). This may be because pharmacological interventions directly affect the closely linked coronary vasculature functions *in vivo* (Beyer *et al.*, 1994) and isolated heart preparations. In many species, ET-1 produces potent and long-lasting constriction of coronary vessels (Clozel & Clozel, 1989; Pernow *et al.*, 1989), producing marked reductions in coronary blood flow and causing associated myocardial ischaemia and even arrhythmias (Ercan *et al.*, 1996). ET_A and ET_B receptor antagonists have been shown to increase and decrease coronary flow respectively in isolated perfused rat hearts indicating the presence of both ET receptors on the coronary vasculature, suggesting an indirect effect of ET-1 on cardiac muscle through modulation of coronary artery tone (Wang *et al.*, 1994). In fact, although clinical studies have not observed coronary vasoconstriction, it has been recognised in humans who are bitten by the Israeli burrowing asp *Actactaspis engaddensis*, a source of SFTX S6c (Weiser *et al.*, 1984).

Although *in vivo* and isolated heart studies have not proved successful in demonstrating the positive inotropic effects in response to ET-1 administration, some *in vitro* preparations of cardiac muscle have managed to show this effect. Observations have shown that nanomolar concentrations of ET-1 can evoke positive inotropic effects on isolated cardiac tissues such as guinea pig atria (Ishikawa *et al.*, 1988), rat atria and ventricular papillary muscle (Hu *et al.*, 1988; Moravec *et al.*, 1989) and rabbit ventricular papillary muscle (Li *et al.*, 1991; Kasai *et al.*, 1994). Studies using human atrial and ventricular myocardial strips (Moravec *et al.*, 1989; Meyer *et al.*, 1996) have observed similar responses. In addition, both Davenport *et*

al. (1989) and Moravec *et al.* (1989) have shown that ET-1 was less effective at causing an increase of contractile force in human and rat ventricle compared to atrial muscle. This difference is likely to be related to the fact that atrial muscle contains more ET receptors than ventricular muscle. However, the identity of the receptor(s) that mediate the response to exogenous ET-1 is unclear. Meanwhile, studies using isolated cardiomyocytes from rabbit and humans have demonstrated positive ET-1 induced contraction (Kelso *et al.*, 2000; Burrell *et al.*, 2000) but suggest that only the ET_A receptors mediate the contractile effect via a PLC pathway.

In addition, separate studies have demonstrated anti- and pro-arrhythmic actions of ET-1. James *et al.* (1994) demonstrated that in isolated ventricular cardiomyocytes, ET-1 acting through ET_A receptors inhibited the PKA-dependent chloride current to protect the ventricle against arrhythmias. Meanwhile, ET-1 induced arrhythmic contractions have been reported in human isolated atrial tissue, however, these effects did not appear to be mediated by the ET_A receptor (Burrell *et al.*, 2000). Paradoxically, high doses of BQ123, the selective ET_A antagonist, have produced arrhythmias while low doses of BQ123 were found to reduce the incidence in the rat model of ischaemia (Garjana *et al.*, 1995). Together, these studies indicate that ET-1 can affect the heart directly and indirectly and is involved in contractility and rhythmicity.

In the heart, ET-1 induces positive inotropic effects by similar cellular mechanisms to those mediating SMC contraction (*Figure 1.5*). Studies have shown that the increased contractile force arises from an accumulation of IP_3 and its associated rise in $[Ca^{2+}]_i$ in rat (Vigne *et al.*, 1989; Kramer *et al.*, 1991), rabbit (Takanashi & Endoh, 1992) and human cardiac tissue (Vogelsang *et al.*, 1994). ET-1 induced DAG production together with Ca^{2+} and phosphatidylserine activates PKC (Takanashi & Endoh, 1991), which can activate targets such as the sodium/hydrogen exchanger (Na^+/H^+X) (Meyer *et al.*, 1996). This leads to an accumulation of Na^+, an increase of intracellular pH, sensitising the contractile proteins and possibly increasing the activity of the sodium/calcium exchanger ($Na^+/Ca^{2+}X$) to remove Na^+ but further increasing $[Ca^{2+}]_i$. In addition, PKC can activate other targets such as nuclear

signalling mechanisms, activator protein (AP-1) and mitogen-activated protein kinase (MAPK), suggesting possible effects on long-term regulation of cellular function.

Vogelsang *et al.* (1994) demonstrated a second signal transduction pathway that inhibits AC activity by a PKC-independent manner, reducing the accumulation of cAMP in human right atrium. A similar mechanism has also been described in rat (Hilal-Dandan *et al.*, 1992) and feline cardiomyocytes (Jones, 1996). Furthermore, multiple G-proteins may be involved in the inotropic response. Ladoux & Frelin (1991) demonstrated that ET-1-induced IP_3 formation involves a PTX-insensitive G-protein, whereas ET-1-induced inhibition of AC involves a PTX-sensitive G-protein (Jones, 1996). It has also been suggested that ET-1 may increase inotropy by enhancing Ca^{2+} responsiveness, with little or no increase in $[Ca^{2+}]_i$ (Paik *et al.*, 1994). This myofilament sensitisation has been demonstrated to act via PKC and the subsequent activation of the Na^+/H^+X (Meyer *et al.*, 1996; Endoh *et al.*, 1998).

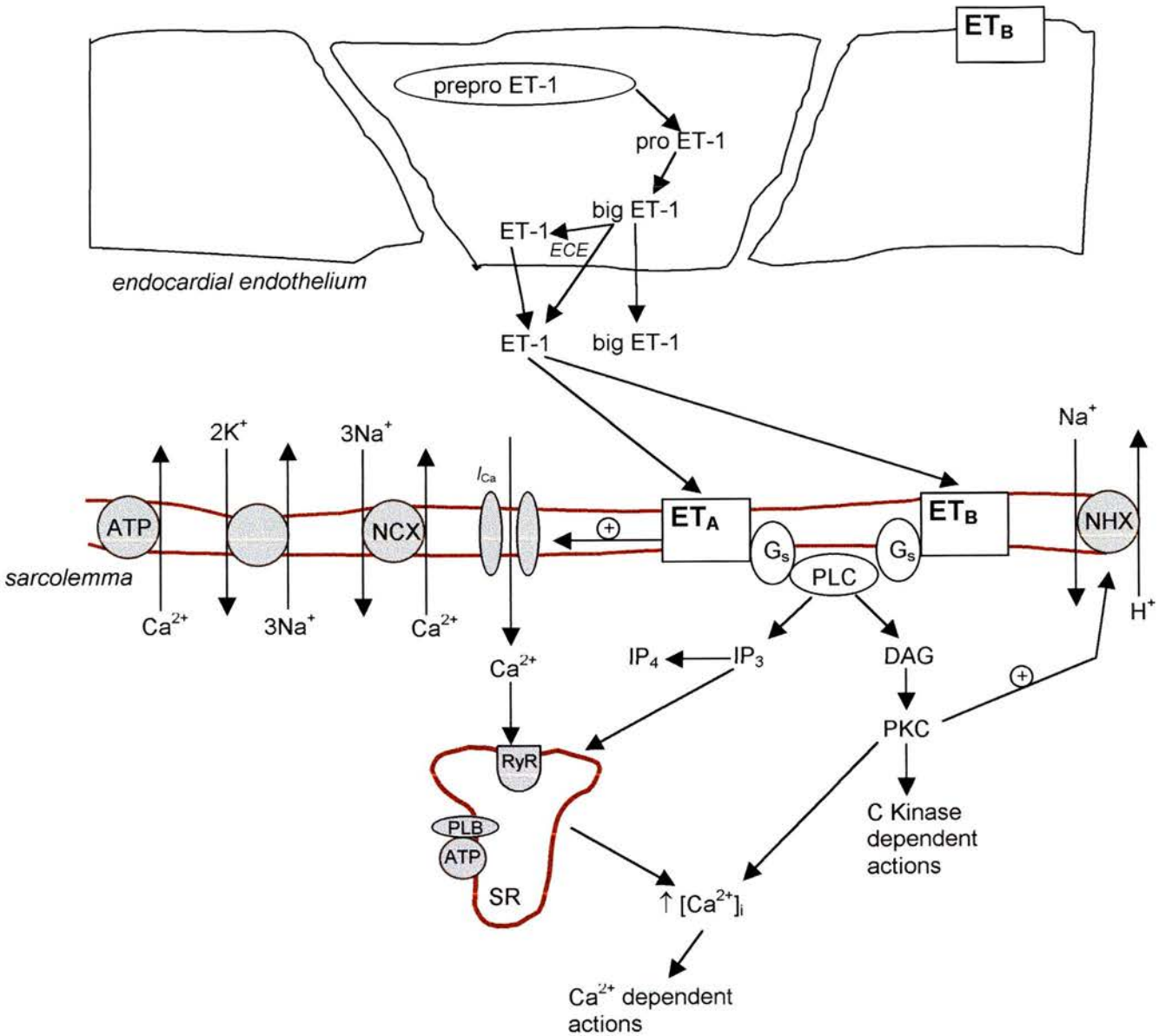


Figure 1.5. The proposed mechanism for the ET-1 mediated increase in contractile force. ATP, ATPase pump; SR, sarcoplasmic reticulum; RyR, ryanodine receptor; PLB, phospholambam; IP_3, inositol triphosphate; DAG, diacylglycerol; PKC, protein kinase C; NCX, Na^+/Ca^{2+} exchange; NHX, Na^+/H^+ exchange; G_s, G-protein; PLC, phospholipase C.

Two classes of voltage-dependent Ca^{2+} channels are exhibited on cardiac myocytes, the L-type channel, which conveys the main depolarising current contributing to the plateau-shape of the action potential and the T-type channel, which is negligible in most ventricular myocytes (as reviewed by Bers, 2002). Channels carrying the I_{Ca} are regulated by a number of second messengers and have been found to be modulated by ET-1. Both stimulation (Lauer *et al.*, 1992; Bkaily *et al.*, 1995) and inhibition (Ono *et al.*, 1994) of the I_{Ca} has been described in response to ET-1. Furthermore, Kelso *et al.* (1998) demonstrated that the ET_A receptor subtype was coupled to a decrease in I_{Ca}, whereas the ET_B receptor was responsible for an increase in I_{Ca} in isolated rabbit ventricular cardiomyocytes. Additionally, Boixel *et al.* (2001) found similar results in human atrial myocytes, suggesting an important mechanism in protecting against arrhythmias and myocardial Ca^{2+} overload. However, evidence has shown that ET_A receptor antagonists such as BQ123 reduced $[Ca^{2+}]_i$ rising in neonatal atrial and ventricular cardiac cells (Touyz *et al.*, 1996), meanwhile, ET-1 has also been shown to enhance Ca^{2+} entry through T-type channels in cultured neonatal rat ventricular myocytes (Furukawa *et al.*, 1992), indicating that the mechanisms underlying the ET-1 receptor-induced $[Ca^{2+}]_i$ rise are not completely understood.

Other actions in the heart include ET-1-induced hyperpolarisation and shortening of action potential duration by inhibition of I_{Ca} and stimulation of the K^+ current (I_K) in isolated guinea pig myocytes (Ono *et al.*, 1994). These effects are coupled to a PTX-sensitive GTP-binding protein and the inhibition of cAMP accumulation, suggesting that the endothelin system has chronotropic and inotropic effects in the heart.

The development of cardiac hypertrophy induced by haemodynamic overload is likely to be triggered by mechanical stress and the involvement of growth factors. As discussed before, stretch of endothelial cells results in a PKC mediated release of ET-1 (see section 1.2.3) and similarly, an increased expression of ET-1 mRNA and stimulated release of ET-1 is observed in response to mechanical stretch of cultured cardiomyocytes (Yamazaki *et al.*, 1996). The contribution of local ET-1 to hypertrophy was demonstrated by an increase in ventricular ET-1 levels during

pressure overload of rat cardiomyocytes, which display a positive correlation with the degree of hypertrophy (Arai *et al.*, 1990). In addition, it has been shown that ET-1 stimulates hypertrophy as indicated by an increase in myocardial cell size, protein synthesis, accumulation of contractile proteins and expression of immediate early genes in cultured rat ventricular myocytes (Shubeita *et al.*, 1990; Ito *et al.*, 1991). The importance of ET-1 stimulation as a paracrine mechanism for the *in vivo* regulation of cardiac growth and hypertrophy was confirmed by Ito *et al.* (1994) in rats with LV overload produced by aortic banding. The study also suggested that the ET_A receptors were involved in the mechanism of cardiac hypertrophy but only during the first week of overload. Similarly, Yamazaki *et al.* (1996) demonstrated that ET-1 induced activation of hypertrophic signals such as Raf-1 kinase (Raf-1) and MAPK were mediated through the ET_A receptor, followed by an increase in protein synthesis in neonatal rat cardiomyocytes. The involvement of the ET_B receptor in mitogenesis has also been demonstrated. Aquilla *et al.* (1996) found it was associated with the activation of three distinct MAPK signal transduction pathways, the extracellular regulated protein kinase (ERK) 2, c-Jun N-terminal kinase 1 (JNK), and p38 kinase. These mitogen-activated protein kinase isozymes are thought to mediate very different biological outcomes and required residues in the c-terminal for coupling the receptor to intracellular kinase pathways. Interestingly, in a previous study in our laboratory, ET_B receptors were increased in the hypertrophic LV post MI in association with ERK and β-myosin heavy chain immunoreactivity (McEwan *et al.*, unpublished observations), indicating that the ET system has remarkably diverse biological properties and actions.

1.3 Heart failure

Chronic heart failure (CHF) is a major public health problem in the UK, affecting a growing number of the population. It is associated with high morbidity and mortality and is a major cause of recurrent hospitalisation. It is associated with activation of compensatory neurohumoral reflexes, including renin-angiotensin-aldosterone system (RAAS) and sympathetic nervous system activation that not only maintain perfusion pressure but also increase peripheral vascular resistance, renal sodium reabsorption and cardiac workload. This leads to a vicious cycle of declining cardiac function and provides a rationale for the current mainstay of treatment, which are directed at preventing sodium and water retention and antagonising the humoral responses that cause peripheral vasoconstriction. The main causes of CHF are ischaemic heart disease (IHD), hypertension, valvular disease and cardiomyopathies such as hypertrophic, restrictive and dilated.

1.3.1 The aetiology of human heart failure

The most common cause of CHF is IHD, which usually follows an acute myocardial infarction (MI), most commonly due to coronary atherosclerosis. LV systolic dysfunction, such as low cardiac output, low ejection fraction and sometimes hypotension, or diastolic dysfunction such as impaired LV filling, high LA pressure and pulmonary oedema, or both, can cause cardiac failure after MI. Patients exhibit exertional fatigue, breathlessness and often symptoms of fluid retention. Prognosis can be predicted from the severity of symptoms, patients with LV ejection fraction (EF) <30% suffer 15-40% mortality at 1 year, compared to less than 5% mortality for those with EF >60%. The main modes of death are sudden arrhythmic death, progressive pump failure and recurrent MI.

Dilated cardiomyopathy (DCM) is the most common cardiomyopathy, which causes include idiopathic, familial (~25% of cases) and myocarditis. It is a heterogeneous cardiac disease characterised by ventricular dilatation and systolic dysfunction. Several genetic studies have recently revealed that mutations in genes for cardiac sarcomere components lead to DCM (Olson *et al.*, 1998; Itoh-Satoh *et al.*, 2002;

Gerull *et al.*, 2002). Hypertrophic cardiomyopathy (HCM) is characterised by myocardial hypertrophy in the absence of an identifiable cause such as hypertension or aortic stenosis. The distribution of hypertrophy may be concentric, asymmetrical or apical. Histologically, HCM is characterised by myofibrillar disarray and myocardial fibrosis. It can be sporadic or hereditary, as a familial predisposition can be identified in 50% of patients and inheritance is usually autosomal dominant. A large number of gene defects have also been identified with HCM and usually affect structural myocyte proteins such as β-myosin, myosin-binding proteins and the troponin subunits. Different disease genes in HCM are associated with different patterns of penetrance and different prognosis (Watkins *et al.*, 1992; Moolman *et al.*, 1997).

Congenital heart disease is characterised by anatomical or structural abnormalities of the heart chambers or great vessels, which may reflect particular deficiencies in neural crest formation. Obstruction of the blood flow across valves or through the great vessels produces a pressure gradient across the lesion, which can lead to the development of hypertrophy or dilation.

Left ventricular hypertrophy (LVH) as an initially useful physiological adaptation to conditions of increasing metabolic and haemodynamic overload is well recognised. In this physiological hypertrophy, as seen in normal growth and increased regular physical exercise, the normal cardiac ultrastructural architecture is preserved. In contrast, pathophysiological hypertrophy is seen in diseased states or in the setting of increased release of factors associated with abnormal cardiac physiology, overexpression of proto-oncogenes and certain genetic mutations. Architectural remodelling comprises of myocyte hypertrophy rather than hyperplasia to match heart size to haemodynamic load. In addition to hypertension, the major aetiologies of LVH include valvular heart disease, HCM, DCM and ventricular septal defects. Among these, chronic severe valvular heart disease and HCM are most likely to result in severe LVH. Thus in diseased states, the limits of LVH are determined predominantly by the degree and duration of haemodynamic pressure and/or volume

overload and any underlying genetic or hereditary susceptibility to cardiac hypertrophy.

1.3.2 Current clinical treatments

Current clinical treatments are directed at correcting the inadequate contractility of the failing heart by reducing workload of the ischaemic myocardium. Patients with signs of fluid retention, including pulmonary oedema, peripheral oedema or elevated jugular venous pressure are generally prescribed diuretics (such as frusemide or bendrofluazide). Increased diuresis results in a reduced blood volume, which is accompanied by an improvement in cardiac performance due to the reduced preload and afterload. However, caution must be taken when using diuretics in combination with other drugs, for example, potassium-sparing diuretics must be used with care when combined with ACE inhibitors as serious renal dysfunction and life-threatening hyperkalaemia may occur. Other side effects of diuretics can include activation of neurohormonal systems, imbalance of electrolytes and hypotension.

ACE inhibitors (such as captopril or ramipril) block the conversion of angiotensin I (Ang I) to Ang II and prevent the degradation of bradykinin by ACE. When added to diuretic therapy, ACE inhibitors reduce the symptoms and signs of heart failure and improve exercise capacity. A number of randomised controlled trials have shown that ACE inhibitors improve ventricular function, improve survival in patients with all grades of heart failure and reduce hospital admissions (as reviewed by Lonn & McKelvie, 2000). In addition, ACE inhibition slows down the progressive deterioration in cardiac status, which is seen in heart failure patients. Common adverse effects associated with ACE inhibitors can include syncope, due to hypoperfusion, hypotension, deterioration of renal function and hyperkalaemia. However, careful initiation of treatment and regular monitoring helps to reduce these problems. Ang II receptor antagonists have similar effects on haemodynamics, neuroendocrine activity and exercise capacity to those of ACE inhibitors (Crozier *et al.*, 1995) and have been used in place of ACE inhibitors when side effects, such as cough are encountered. Pitt *et al.* (1997) evaluated the effects of the ACE inhibitors captopril, compared to Ang II receptor antagonist losartan (in addition to diuretic and

digoxin therapy) and reported a significantly lower mortality in the losartan group. However, losartan was not superior to captopril in respect of symptomatic status or hospitalisation rates for worsening heart failure. Aldosterone antagonists such as spironolactone have also been shown to have a beneficial effect on both mortality and morbidity in heart failure and a substudy has shown that these effects are at least partly linked to a reduction in cardiac fibrosis (as reviewed by Delcayre & Swynghedauw, 2002).

Positive inotropes such as digoxin improve the symptoms and signs of heart failure, the quality of life and the exercise tolerance of patients with mild, moderate, or severe heart failure. In addition, digoxin increases the delivery of sodium to the distal tubules, leading to the suppression of renin secretion from the kidneys and activates the parasympathetic nervous system to increase vagal tone (as reviewed by Dec, 2003). However, the major pharmacokinetic drawback of digoxin is its narrow dose-response relationship and it is administered at a fixed dose (0.125-0.25 mg daily in the majority of patients) to avoid toxicity.

Beta adrenoceptor antagonists (beta-blockers; such as carvedilol or metoprolol) decrease the three major determinants of myocardial oxygen demand, heart rate, contractility and systolic wall tension, allowing improvement in LV function. Originally, β-adrenoceptor antagonists were used to treat angina and hypertension, however, clinical trial evidence suggested that patients with clinically stable, mild to moderately symptomatic heart failure caused by LV systolic dysfunction may benefit from treatment (Packer *et al.*, 1996; CIBIS-II Investigators, 1999; Swedberg *et al.*, 1999). However, major side effects such as hypotension, fluid retention and bradyarrhythmias are often related to the use of β-adrenoceptor antagonists. In addition, conventional doses can cause worsening of heart failure, therefore, small doses are started cautiously and carefully observed. Controversy still surrounds the management of heart failure and despite all of the recent advances in medical and other therapies, there is still room for improvement.

1.3.3 Animal models of heart failure

Animal models of heart failure have provided a useful starting point for the investigation of cardiac diseases and have aided in the development of therapeutic drugs (as reviewed by Hasenfuss, 1998a). The most widely used model in cardiovascular research is LV coronary artery occlusion (CAL), as described by Pfeffer *et al.* (1979), which results in MI. The popularity of this model stems from the fact that it is the only animal model of heart failure to closely mimic IHD in humans (as reviewed by Arnolda *et al.*, 1999). Rats, which survive the acute phase of MI, develop progressive LV and RV hypertrophy and remodelling. Failure is associated with LV dilatation, reduced systolic function and increased filling pressures. Altered haemodynamics and neurohumoral activation, similar to those seen in patients with CHF are also observed. Another technique, aortic banding, creates haemodynamic overload which leads to the development of LV hypertrophy (Ito *et al.*, 1994). This model is also associated with an elevation in activity of the renin-angiotensin system. In addition, CHF induced by rapid ventricular pacing displays characteristic reductions in cardiac output and mean arterial pressure, increased pulmonary capillary wedge pressure and increased levels of plasma norepinephrine, aldosterone and the hypertrophic marker atrial natriuretic peptide (Ohnishi *et al.*, 1998). Models of hypertrophic cardiomyopathy have been developed experimentally to study the functional aspects of myocardial hypertrophy and more recently molecular biology techniques have engineered more refined animal models of hypertrophic cardiomyopathy (Tardiff *et al.*, 1998; Yang *et al.*, 1998). However, it is accepted that no animal model can completely mimic human heart failure.

1.3.4 Heart failure and the endothelin system

1.3.4.1 Elevated levels of ET-1 in human heart failure

Plasma levels of the ET-1 precursor, big ET-1 are increased in patients with CHF, indicating an increase of ET-1 synthesis. These levels are found to correlate with the degree of haemodynamic and functional impairment (Pacher *et al.*, 1993; Wei *et al.*, 1994). Plasma concentrations of mature ET-1 peptide are also elevated in more

severe cases, when renal failure and pulmonary congestion are associated with reduced clearance of ET-1. A two-threefold increase of plasma ET-1 concentration has been reported in patients with CHF compared to healthy individuals (McMurray *et al.*, 1992) and such levels are capable of producing systemic vasoconstriction and reduction of the cardiac index (Cowburn *et al.*, 1998). However, the clearest indication that ET-1 is a mediator, rather than a marker of CHF comes from ET-receptor antagonist studies (see section 1.3.4.3 below).

The expression and distribution of the ET-1 system has been studied in human heart failure. Endothelial cells of ventricular tissue from patients with IHD or DCM have previously been shown to express ET-1 mRNA and immunoreactive ET-1 (Plumpton *et al.*, 1996). In addition, expression of ET-1 immunoreactivity and ppET-1 mRNA have been localised to vascular and endocardial endothelial cells, as well as cardiomyocytes from explanted hearts of patients with end-stage heart failure (Giaid *et al.*, 1995). The most intense staining for ET-1 has been identified in myofibrils, which are located in areas of granulation tissue surrounding the infarcted area. Furthermore, in addition to increased ET-1 mRNA, a recent study reported the upregulation of ECE-1 mRNA in failing human myocardium, supporting the increase in ET-1 synthesis (Morawietz *et al.*, 2002). However, a contrasting study demonstrated that immunoreactive ET-1 displayed similar intensities in both healthy and CHF ventricular myocardium (Wei *et al.*, 1994).

1.3.4.2 Altered expression of ET receptors in human heart failure

ET signalling is activated in failing human hearts and may contribute to progressive myocardial dysfunction and remodelling. However, the behaviour of ET receptor subtypes in failing human hearts is not well understood. Both receptor genes are expressed in the failing myocardium, with the ET_A receptor the dominant subtype but no clear pattern has emerged for altered expression levels relating to aetiology. Pönicke *et al.* (1998) demonstrated elevated ET_A receptor density in the LV of human end-stage failing myocardium. Similarly, ET_A upregulation in the LV myocardium of hearts in end-stage failure due to DCM compared to non-failing was demonstrated by Pieske *et al.* (1999), while ET_B receptor expression remained

unchanged. In contrast, Zolk *et al.* (1999) reported a significant decrease of ET_B receptor mRNA in DCM samples compared to non-failing and no significant change in expression of the ET_A receptor, or ET-1 mRNA. Knowledge of ET receptor density in heart failure is of importance as the actions of ET-1 during the progression of CHF may depend on the cardiac ratio.

1.3.4.3 Endothelin antagonists and human heart failure

CHF is clearly one of the most important pathologies that could potentially benefit from treatment with ET receptor antagonists (Duchman *et al.*, 2000). Clinical studies have shown that short-term therapy with the dual ET_A/ET_B receptor antagonist bosentan improves systemic and pulmonary haemodynamics substantially in conventionally treated patients with symptomatic severe CHF (Kiowski *et al.*, 1995; Sutsch *et al.*, 1998). In addition, selective ET_A receptor blockade has also been reported to induce favourable effects in patients with CHF (Cowburn *et al.*, 1998). These studies have established that ET-1 exerts measurable haemodynamic effects in heart failure. However, whether selective ET_A receptor antagonists are as efficient as mixed antagonists in CHF remains to be determined, as they have not been compared directly in the same study. It is suggested that theoretically, selective ET_A receptor antagonists may offer advantages over mixed ET_A/ET_B receptor antagonists. ET_B receptors are known to evoke vasodilatation and are involved in clearance of ET-1. Therefore, blockade of the ET_B receptors may exacerbate the already reduced pulmonary clearance of ET-1, which may contribute to the elevated circulating ET-1 levels in CHF (Dupuis *et al.*, 1998).

1.3.4.4 The endothelin system in experimental models of heart failure

Elevated circulating levels of plasma ET-1 and bigET-1 concentration have been reported in some experimental models of heart failure and like in the human, correlate positively with disease severity during the progression from MI to CHF. Previous studies have demonstrated that ET-1 mRNA levels are significantly elevated in the LV CHF rats (Yorikane *et al.*, 1993; Sakai *et al.*, 1996b), peaking 1 week after MI and declining thereafter (Øie *et al.*, 1997; Tønnessen *et al.*, 1997).

Studies in our laboratory, using a model of less severe heart failure did not find ET-1 mRNA to be changed at 12 weeks post MI (Sherry, 2000), suggesting that upregulation of ET-1 expression may be time dependent or related to disease severity.

Upregulation of ET receptor density has also been reported in the LV of MI induced CHF rats. Tønnessen *et al.* (1997) demonstrated that both ET_A and ET_B receptor subtypes tended to be upregulated in the LV 1 week following MI. In addition, similar studies have also demonstrated elevated levels of both receptors 3 weeks (Sakai *et al.*, 1996a; Kobayashi *et al.*, 1999), 4 (Picard *et al.*, 1998) and 12 weeks (Sakai *et al.* 1996b) after CAL surgery. Meanwhile, in our laboratory, a selective upregulation of ET_B receptor mRNA was expressed in the failing LV of rats during the 5-12 weeks following CAL surgery (Sherry, 2000), possibly attributed to the model exhibiting less severe heart failure. As discussed before, the abundance and/or sensitivity of the ET receptors may play an important role in the actions of ET-1 during the progression of heart failure. However, to date the function of the receptor subtypes in failing and non-failing myocardium is not clear. It is likely that upregulation of endothelin pathways is beneficial in providing short-term inotropic support for the failing myocardium in which β-adrenergic responsiveness is frequently attenuated. However, as heart failure progresses, ET system activation becomes a maladaptive response, as identified from the results of ET antagonists, which indicate the symptomatic benefits of ET blockade.

Long-term treatment of CHF rats with the mixed ET_A/ET_B receptor antagonist bosentan (Fraccarollo *et al.*, 1997; Mulder *et al.*, 1997; Øie *et al.*, 1998) or selective ET_A receptor antagonist BQ123 (Ito *et al.*, 1994; Sakai *et al.*, 1996a, 1996b), has previously been reported to improve the survival of rats post MI by improving cardiac haemodynamic function and limiting LV dilatation. Both receptors are reportedly involved with the hypertrophic response to ET-1 in experimental heart failure (Mullan *et al.*, 1997). However, if cardiac ET receptors are upregulated to provide inotropic support to the failing myocardium, the beneficial effects of ET receptor antagonists must be due to the blockade of the systemic effects of ET-1

action. To date, the contribution of ET receptor subtypes to cardiac contraction in the failing myocardium has not clearly been defined. Therefore investigation would provide a better understanding of the influences affecting cardiac function in health and disease and identify whether cardiac ET receptors play a central role.

1.4 General aims of the study

ET-1 is considered to be involved in the development and progression of heart failure as levels of the endothelium-derived vasoconstrictor-inotropic peptide are increased in heart failure and correlate to prognosis (Rubanyi & Polokoff, 1994; Love & McMurray, 1996). Upregulation of ET-1 system components has been demonstrated in both heart failure patients (Pieske *et al.*, 1999; Zolk *et al.*, 1999) and experimental animal models of heart failure (Kobayashi *et al.*, 1999), with selective upregulation of the ET_B receptor having been previously demonstrated in our laboratory (Sherry, 2000). However, a distinct lack of clarity exists regarding the contribution of the receptor subtype(s) in the regulation of cardiac contractility in health and disease states.

The main aims of this thesis were to investigate the function of the ET receptor subtypes in relation to cardiac contractility in the CAL rat *in vivo* and *in vitro* and to identify whether upregulation of the ET_B receptor contributes to the contractile behaviour of failing myocardium *in vitro*. In addition, selective or dual ET_A/ET_B receptor blockade of endogenous ET-1 was investigated in the normal rat myocardium with the aim to better define the receptor subtype(s) responsible for mediating the inotropic response to ET-1. Echocardiography was used to compare the LV contractility of sham-operated rats with CAL rats *in vivo*. Additionally, control animals following acute or 1-week chronic administration of the dual ET_A/ET_B receptor antagonist bosentan were compared with placebo treated animals to further investigate the contribution of endogenous ET-1 to cardiac contractile behaviour. Finally, transmural expression of ET system components, cytoskeletal proteins and genes associated with hypertrophy were explored in human failing LV myocardial samples to determine if distribution related to wall stress and myocardial stiffness.

Chapter 2

Methods

This chapter describes the experimental techniques used in one or more of the subsequent chapters in this thesis. All experiments using animals were carried out in accordance with the Animals (Scientific Procedures) Act 1986.

Informed consent was obtained from patients to allow storage and use of human myocardial tissue as part of medical research. Samples were acquired between October 1997 and February 1998 from patients undergoing transplant surgery.

2.1 Rat coronary artery ligation model of CHF

2.1.1 Introduction

In cardiovascular research, animal models provide a useful tool for the investigation of cardiovascular diseases and have aided in the development of therapeutic drugs. The characteristics required by an ideal model are i) to provide an adequate likeness to human disease, ii) allow studies in chronic, stable disease, iii) produce symptoms which are predictable and controllable, iv) to be relatively inexpensive and satisfy technical and animal welfare considerations and v) allow the measurement of relevant functional parameters (Doggrell & Brown, 1998). A variety of techniques are currently available for the induction of experimental heart failure in a range of species (reviewed by Doggrell & Brown, 1998; Hasenfuss, 1998a). However, it is acknowledged that the complex pattern of human heart failure cannot be entirely mimicked by any one animal model.

Myocardial infarction (MI) following coronary artery ligation (CAL) in rats is the most common rat model of heart failure (HF). Selye *et al.*, (1960) were the first to describe the CAL technique in the rat and subsequently, Pfeffer *et al.* characterised the model in 1979 (Pfeffer *et al.*, 1979). Left ventricular (LV) MI occurs as a consequence of complete occlusion of the left coronary artery and as a result, LV function is impaired. LV dilatation, increased filling pressures and reduced systolic function are associated with the progression of LV dysfunction (Litwin *et al.*, 1994) as well as changes in neurohormonal activation, similar to those seen in patients with CHF (Hodsman *et al.*, 1988).

Several limitations exist with all HF models regarding the differences in myocardial function compared to the human heart. Indeed, since CAL surgery is carried out on essentially healthy coronary arteries and myocardium, it is accepted that the rat model is not entirely analogous to the pathogenesis of coronary artery disease or infarction in humans (Pfeffer *et al.*, 1979). A high initial mortality rate and variable size of infarct present further problems for the CAL model. However, despite the disadvantages, the rat CAL model of CHF has proven to be useful in evaluating particular aspects of failure, has clinical relevance and has predicted results of pathophysiological and pharmacological studies in man. It is for this and the aforementioned reasons that the chronic infarcted rat heart was used as a model in this thesis.

2.1.2 Induction of myocardial infarction

MI was produced in male Wistar rats (200-250g, Charles River, France) by occlusion of the left anterior descending coronary artery according to the method described by Selye *et al.* (1960) and modified by Pfeffer *et al.* (1979). The animals were anaesthetised with sodium pentobarbital (60 $mg \cdot kg^{-1}$, i.p., Sagatal, Rhone Merieux Ltd., Essex, U.K.). Prior to surgery, the left side of the chest was shaved and sterilised using 70% alcohol. The animals were placed on a thermostatically controlled heat mat (37°C) and intubated with a plastic cannula (1.65mm diameter, 5FG, Portex Ltd., Kent, U.K) using a guide wire. Mechanical ventilation with oxygen enriched room air was provided by use of a small rodent ventilator (Harvard Apparatus Ltd., Kent, U.K.) at a rate of 60 cycles per minute and a tidal volume of 1ml/100g body weight.

An incision (~2 cm) was made into the left side of the chest, parallel to the direction of the ribs. The underlying muscles were carefully separated and held in position with metal clips to expose the ribs. A left thoracotomy was performed between ribs 4 and 5, with care being taken not to damage the lungs while making the incision. The left lung was then gently pushed down and away from the heart using a triangular piece of synthetic surgical sponge (Fine Science Tools, InterFocus Ltd., Suffolk, England). A hole was made in the pericardium using a blunt pair of scissors and the

heart was gently and rapidly manipulated out of the thoracic cavity by applying pressure to the right hemithorax. A 5-0 silk suture (10mm round bodied, Ethicon Ltd., Edinburgh, U.K.) was passed around the proximal part of the left anterior descending coronary artery, below the left atrial appendage. The heart was then swiftly returned into the thorax. The animal was allowed 10 minutes to recover from the exteriorisation before the suture was tied. Sham-operated animals were subjected to the same protocol, except that the snare was not tied. Animals were randomly assigned to groups. The sponge used to collapse the lung was removed and a plastic cannula (0.75mm diameter, 3FG, Portex Ltd.) connected to a 2.5ml syringe was placed in the thorax before closure with a 3-0 silk suture (16mm round bodied, Ethicon Ltd.). Before tying the last suture, the chest was gently squeezed to expel air from the thoracic cavity. The overlying muscles were returned to their original position. Any remaining air within the thorax was removed by way of the cannula and syringe, allowing the rat to resume spontaneous respiration. The cannula was removed and the skin was sutured using a 3-0 mersilene suture (25mm round bodied suture, Ethicon Ltd.). The animal was maintained on the ventilator for a further 15 minutes and then moved to a recovery box, placed on a heated blanket (37°C), where it was positioned near a 100% oxygen source. When the rat showed signs of recovery, the tracheal cannula was removed. Once the animals had fully recovered from the anaesthesia they were given buprenorphine hydrochloride ($0.05mg \cdot kg^{-1}$, Vetergesic, Redcut & Coleman, U.K.) subcutaneously and returned to their cages. Analgesia was also administered the following morning. All rats were housed in climate-controlled conditions with a 12-hr light-dark cycle and free access to normal rat chow and drinking water. CAL surgery was performed by Dr. Gillian Gray.

2.1.3 In vivo characterisation

CAL was used to generate animals for two heart failure studies. Group 1 animals were used 15 weeks post surgery to investigate the activation of the ET system, while group 2 were used 12 weeks after surgery to explore the left ventricular pressure-dimension relationship of the failing rat heart.

2.1.3.1 Fifteen week ET system activation study

2.1.3.1.1 Haemodynamic measurements

Fifteen weeks after surgery, CAL (n=10) or sham-operated (n=10) rats were weighed, anaesthetised with sodium pentobarbital (60 $mg \cdot kg^{-1}$, i.p. Sagatal) and placed on a thermostatically controlled heating mat (37°C) to maintain body temperature. The neck area was shaved and an incision was made from the mandible to the sternal notch. The right carotid artery was located, isolated and cannulated with a fluid filled (heparin, Multiparin, CP Pharmaceuticals, Wrexham, U.K. diluted to 100 $U \cdot ml^{-1}$ in saline) plastic catheter (0.75mm diameter, 3FG, Portex, Ltd.) attached to a pressure transducer. After an initial stabilisation period (5 minutes), arterial blood pressure was measured. The catheter was then carefully inserted via the carotid artery into the left ventricle (LV) to continually record LV pressure. Data was recorded on an Apple Mac computer via a MacLab (version 3.4/e) data analysis system (AD Instruments, Hastings, U.K.). A representative trace of arterial blood pressure and LV pressure measured from a CAL rat is shown in *Figure 2.1*. From *Figure 2.1* it can be seen that as the catheter was advanced into the LV, an increase in the pressure pulse was obtained. Mean arterial pressure (MAP), LV systolic (LVSP) and end-diastolic pressure (LVEDP) values were measured by calculating the average pressure readings from no less than 10 pressure spikes along the respective traces. Measurements of heart rate (HR), the maximal rate of rise and maximal rate of decline of LV pressure (dP/dt_{max} / dP/dt_{min}) were also obtained. Ligated rats with LVEDPs ≥ 10mmHg were considered to have LV dysfunction.

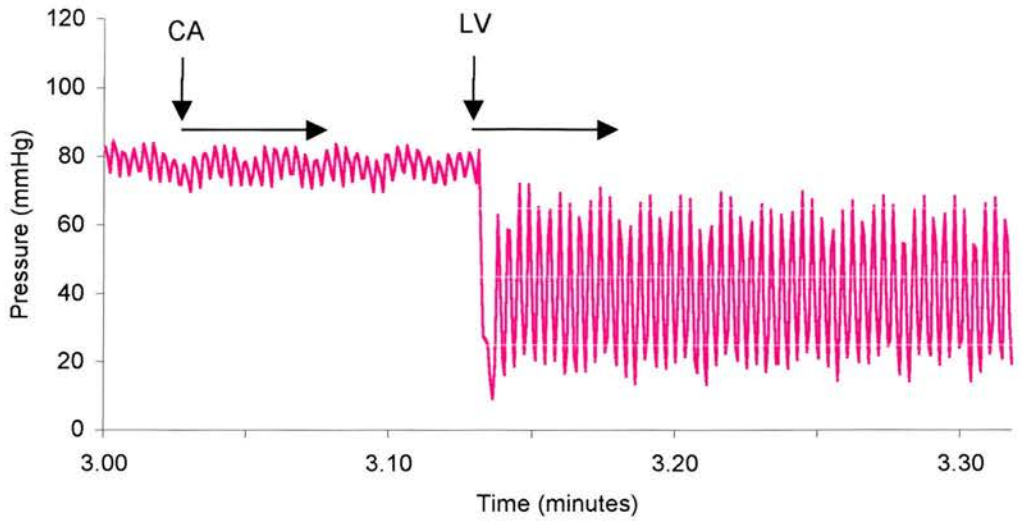


Figure 2.1. A sample trace of arterial blood pressure measured from within the carotid artery (CA) and left ventricular (LV) pressure measured from a CAL rat.

2.1.3.1.2 Tissue harvesting

Following exsanguination, the heart was removed and briefly placed in Tyrode's solution (room T°C: see section 2.4 for composition) where it was permitted to beat four or five times to expel the blood. It was then transferred into an ice-cold cardioplegic solution (Tyrode's solution containing 30mM 2,3-butanedione-monoxime, BDM), causing the heart to cease contractile activity and kept on ice while the other organs were removed, rinsed in Tyrode's, blotted and weighed. Using a stereomicroscope (Leica $MZ7_5$, Leica, Solms, Germany), the atria and large vessels were removed and the heart was weighed. The right ventricle (RV) was then opened along the septum to expose the papillary muscles, which were carefully dissected out for functional studies (see Section 2.2). Throughout the dissection period, the heart remained immersed in ice-cold Tyrode's-BDM solution. The use of BDM during dissection has been shown to protect the myocardium from cutting injury and its effects are fully reversible after washout (Mulieri *et al.* 1989). BDM is a quick-acting, reversible inhibitor of cardiac contractility. It exerts a direct inhibitory effect on cross-bridge interactions and decreases the sensitivity of the contractile proteins to Ca^{2+} thereby reducing contractures (Mulieri *et al.* 1989).

Tissue samples (approximately 100mg) of the RV free wall, LV free wall and the interventricular septum were taken, snap frozen in liquid nitrogen and stored at -70°C for subsequent gene expression studies (see section 2.3). In the case of CAL hearts, the infarcted area (scar tissue) was excised from the non-infarcted LV, carefully avoiding contamination of viable myocardial tissue with necrotic tissue.

2.1.3.1.3 Assessment of infarct size

Due to the dissection of the heart for the removal of papillary muscles, an alternative method for analysing infarct size was employed. The area of infarcted ventricle was placed under a CCD video camera module (Kontron Elektron Images, Carl Zeiss vision GmbH) and the images were viewed on a computer. The area of the infarct was measured using Image Analysis software (Carl Zeiss KS 300 version 3.0).

2.1.3.2 Twelve week Pressure-Dimension study

2.1.3.2.1 Haemodynamic and echocardiographic measurements

Twelve weeks following CAL (n=12) or sham-operation (n=6), rats were weighed and anaesthetised in a chamber with 4% halothane (Halothane-vet, Merial Animal Health Ltd., U.K.) vaporised in a mixture of O_2 (2 l·min^{-1}) and N_2O (0.4 l·min^{-1}) to achieve unconsciousness. The animal was then transferred to a thermostatically controlled heat mat (Harvard Apparatus Ltd.) and body temperature was monitored by a rectal probe and maintained at 37 ± 0.5°C. Anaesthesia was delivered via a nasal cone (reduced to 3% halothane in gas mixture) and the neck, chest and left groin areas were shaved. Once fully unconscious, the left femoral vein was located, isolated and cannulated with a fluid filled (heparin, Multiparin, CP Pharmaceuticals, diluted to 100 U·ml^{-1} in saline) plastic cannula (0.75mm diameter, 3FG, Portex Ltd.) for the infusion of drugs. Subsequently, the right carotid artery was isolated and cannulated with a high-fidelity micromanometer-tipped catheter (SPR-407, size 2F, Millar Instruments Inc., Houston, TX, U.S.A.) connected to a control unit (TCB-500, Millar Instruments Inc.). Halothane anaesthesia was reduced to a level that maintained unconsciousness (usually 1.5%) and after a stabilisation period,

measurements of arterial pressure and HR were recorded. The Millar catheter was then carefully advanced through the aorta into the LV to record LV pressure. All measurements (as before, see section 2.1.3.1.1) were recorded on a laptop computer using a Powerlab system. The Millar control unit was connected to a data acquisition system which consisted of a Powerlab (Powerlab /8sp, AD Instruments), which in turn was connected to a DELL laptop computer (model PP01X, Inspiron 8000) with CHART v4.1 software (AD Instruments).

An incision was made down the midline of the thorax and the skin on the left side was carefully pulled back by blunt dissection to expose the underlying pectoral muscles. Initial investigation showed that better images were obtained this way as scar tissue obscured the image in some animals. The rat was then placed in the left lateral position and the chest smothered with acoustic gel (Clear Image medical ultrasound couplant, Diagnostic Sonar Ltd., Livingston, U.K).

Transthoracic echocardiography was performed using DIASUS Ultrasound Equipment (Dynamic Imaging, Livingston, U.K.) to assess the position of the catheter and to set the depth and focal length of the scan. Care was taken to avoid excessive pressure on the thorax. Using a 128-element probe (10-22MHz) with DIASUS P5.26 software (Dynamic Imaging), the depth was set at 30mm with 1 focus line. On this setting the image was acquired over 0.775 seconds. To obtain an echo image and a synchronised LV pressure recording, a trigger was attached from the ultrasound unit to the Powerlab and the CHART software set to capture catheter data only during this triggered 0.775s fixed period. A representative scan of two-dimensional long and short-axis b-mode images of LV dimensions are shown in *Figure 2.2.* From *Figure 2.2* it can be seen that the anterior and posterior LV wall boundaries are well defined. The Millar catheter can also be seen in the aorta, with the tip in the LV (*Figure 2.2a*). The corresponding catheter data (4 - 5 cardiac cycles; see *Figure 2.3*) was inspected to avoid recording ectopic beats. Several image and pressure sets were captured during the different afterload states.

(i) Baseline measurements

Two-dimensional long-axis b-mode images of the LV were obtained and captured at baseline conditions before administration of any experimental drugs. The echo images were played back to ensure good quality before saving. Subsequently, short axis b-mode images were obtained at the level of the papillary muscles.

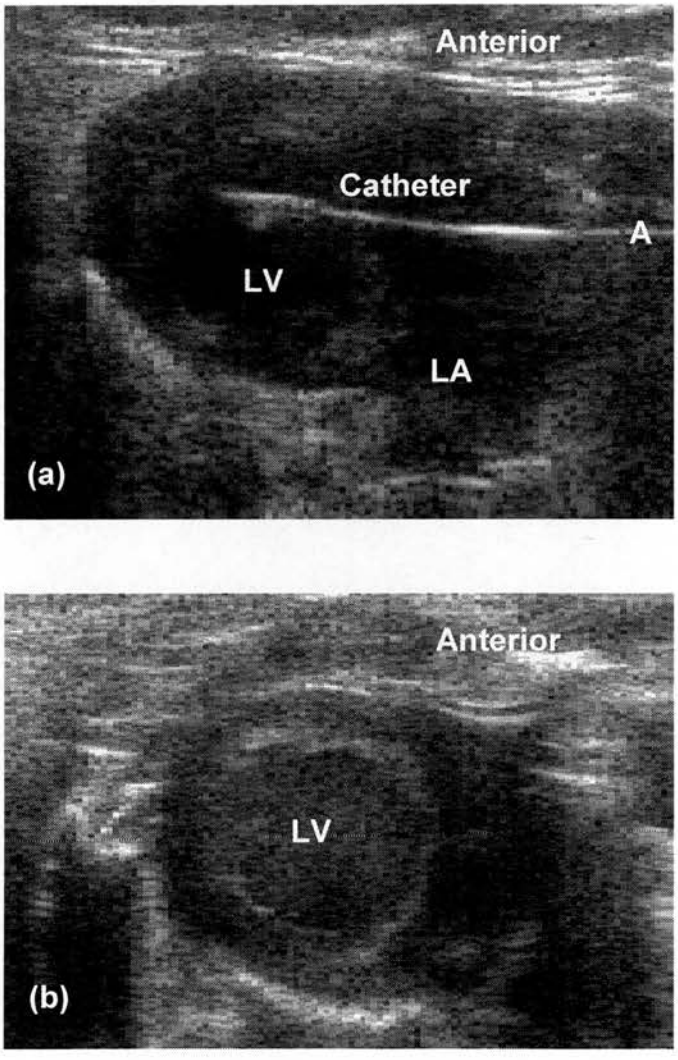


Figure 2.2. Two-dimensional images of (a) long-axis and (b) short-axis b-mode transthoracic echocardiographic scans. LV, left ventricle; LA, left atrium; A, aorta.

(ii) Sodium Nitroprusside infusions

The venous cannula was connected to an infusion pump (Treonic IP4H Syringe pump, Vickers Medical, Stirling, U.K.) via a three-way tap. Sodium nitroprusside (SNP, Sigma, 25 $\mu g \cdot ml^{-1}$ dissolved in saline), a nitric oxide donor drug, was infused to induce a desired systolic pressure drop of 20-30mmHg less than baseline (usually between 1 - 5 $\mu g \cdot kg^{-1} \cdot min^{-1}$). Images were captured at 2-3 minute intervals to obtain a range of pressure changes (usually 10mmHg increments). Short-axis b-mode images were also recorded. Heparinised saline (100 $U \cdot ml^{-1}$) was then used to flush the catheter and a recovery period of 20 minutes was allowed, starting at the end of the infusion. LV pressure returned to baseline levels within 5-10 minutes.

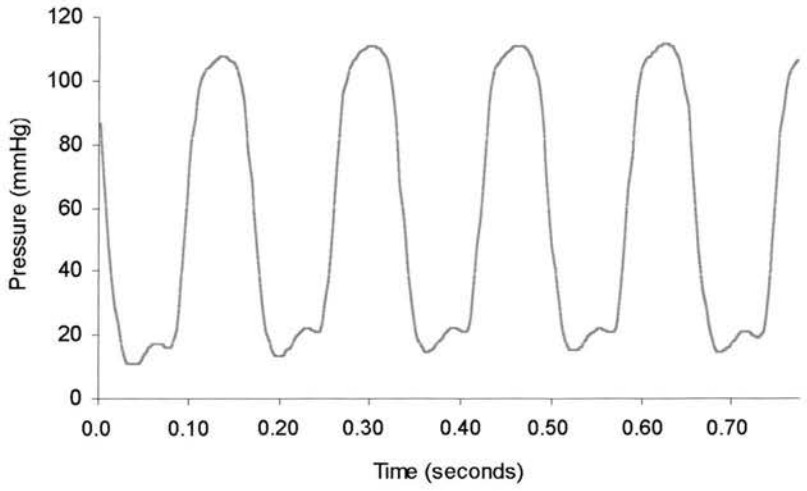


Figure 2.3. A pressure-transducer reading from within the left ventricle, acquired over 0.775 seconds to correspond with an echocardiographic scan.

(iii) Phenylephrine infusions

Following the recovery period, the α_1-adrenergic receptor agonist phenylephrine (PE, Sigma, 0.2 $mg \cdot ml^{-1}$ dissolved in saline) was infused to induce a desired increase in systolic pressure of 20-30 mmHg greater than baseline (usually between 13 - 40 $\mu g \cdot kg^{-1} \cdot min^{-1}$). Images of both long and short axis were taken in b-mode.

2.1.3.2.2 Tissue harvesting

Organs were removed and rinsed in Tyrode's-BDM solution (see section 2.4 for composition), prior to being blotted and weighed. The CAL hearts were sectioned apex-base through the infarcted region. Half was placed in 10% formalin and the remaining tissue divided into LV, RV and septum. Myocardial samples and the kidneys were snap frozen in liquid nitrogen and stored at -70°C for future molecular analysis.

2.1.3.2.3 Histological analysis

As a result of chronic left coronary artery occlusion, a collagen rich scar developed in the infarcted zone of the LV. Using van Gieson's stain for collagen (see section 2.4), infarct size in relation to the LV free wall was measured. Hearts were cut along the apex-base axis and placed in 10% formalin. After 24 hrs samples were taken out and immersed in 70% alcohol. Subsequently, 24 hrs later they were transferred into 50% alcohol before being processed and embedded in paraffin wax. Briefly, the samples were dehydrated by immersion in ascending alcohol concentrations and then into xylene. The xylene was then replaced with paraffin wax under vacuum conditions. The resulting blocks were sliced in 3μm sections and mounted on TESPA (3-Aminopropyltriethoxy-saline, Sigma) coated microscope slides (see section 2.4). After drying in an incubator (37°C) overnight, sections were de-waxed in xylene (10 minutes), sequentially taken through descending alcohol concentrations (100, 90 and 70%, 2 minutes each) and washed in water. Subsequently, the sections were dipped in Celestine Blue nuclear stain (Sigma) for 4 minutes. After rinsing with water, slides were treated with van Gieson's collagen stain for 3-5 minutes and rinsed briefly with water. The sections were then dehydrated by going through an ascending alcohol series (70, 90 and 100%, 3 minutes each) to xylene (10 minutes). Finally the slides were taken individually, dried and mounted with DPX medium (BDH Laboratory Supplies, Dorset, U.K) and coverslips.

Using a method previously described by Mulder *et al.* (1997), the infarct size was measured. Briefly, the stained sections were placed under a CCD video camera

module (Carl Zeiss Vision) and the images were viewed on a computer. The epicardial and endocardial circumferences of the infarcted tissue and of the LV free wall were determined with Image analysis software (Carl Zeiss Vision, KS 300, version 3.0). Infarct size was calculated as (endocardial + epicardial circumference of the infarcted LV tissue) / (endocardial + epicardial circumference of the free LV wall) and expressed as a percentage.

2.2 Pharmacological studies of isolated rat right ventricular papillary muscles

2.2.1 Introduction

In vitro studies, such as isolated cardiac muscle preparations have proven to be a powerful and reliable tool in the evaluation of functional changes in contractile parameters both in human and in animal, normal and failing myocardium. For experimental purposes, papillary muscle preparations are simple to dissect and mount, require little specialised apparatus and will contract in a reliable manner in response to a stimulus. Although isometric contraction is not physiological, it is roughly analogous to isovolumetric contraction *in vivo* and provides a state for normalisation. Papillary muscles are convenient for the purpose as their fibres run a fairly straight course. Consequently, the technique was employed in this thesis to investigate the effects of drugs and other interventions on cardiac muscle. However, it is acknowledged that isolated preparations of heart muscle have some disadvantages due to i) cessation of its circulation, ii) one end of the preparation being crushed, iii) a reduction in the range of hormonal and autonomic transmitters which reach it and iv) being immersed in a solution that is only an approximation of normal extracellular fluid (for review, see Allen, 1983). On balance, it is a suitable preparation for measurements of contractile behaviour and has a small enough cross section for adequate oxygenation.

2.2.2 Preparation of rat right ventricular papillary muscles

The heart (from section 2.1.3.1) was pinned out on a silicon-coated (Sylgard 184 silicone elastomer, Dow Corning Corporation) dissecting dish containing ice-cold

cardioplegic Tyrode's-BDM solution. The right ventricle (RV) was opened under a dissecting microscope and suitable papillary muscles were isolated by first dividing the chordae tendineae at the tip of the muscle, then freeing the muscle base along with a small amount of surrounding myocardium from the ventricular wall. As far as possible, only long, thin, uniformally cylindrical muscles were used. The muscular end of the papillary was fixed to a muscle holder with a spring clip and the tendinous end was tied with a 4-0 surgical silk (Davis and Geck, Hampshire, U.K.) and connected to an isometric force transducer (Fort 10, World Precision Instruments Inc., U.S.A.) via a stationary hook. All dissection and manipulation was carried out in ice-cold Tyrode's-BDM. The preparation was vertically mounted in a 7ml organ chamber (World Precision Instruments Inc., U.S.A.), superfused with carbogen-bubbled (95% O_2: 5% CO_2) cardioplegic solution (37°C) and allowed to stabilise for 20 minutes (see *Figure 2.4*). The passive length-tension relationship was then investigated by progressively stretching the muscle via a micromanipulator, 0.1mm increments / minute. Once achieving L_{max} (passive), the tension was released and the cardioplegic solution was replaced with Tyrode's solution (same composition but without BDM, 2 rinses). The preparation was then stimulated by 2ms square wave pulses at 3Hz, at a voltage 20% above threshold and left to equilibrate for 30 minutes. Following stabilisation, the active-length tension relationship was evaluated by progressively stretching the muscle until an additional stretch did not produce an increase in the developed force; this length was taken as L_{max} (active). The pharmacological protocols applied subsequent to stabilisation are described in the relevant sections of Chapter 3 and Chapter 4.

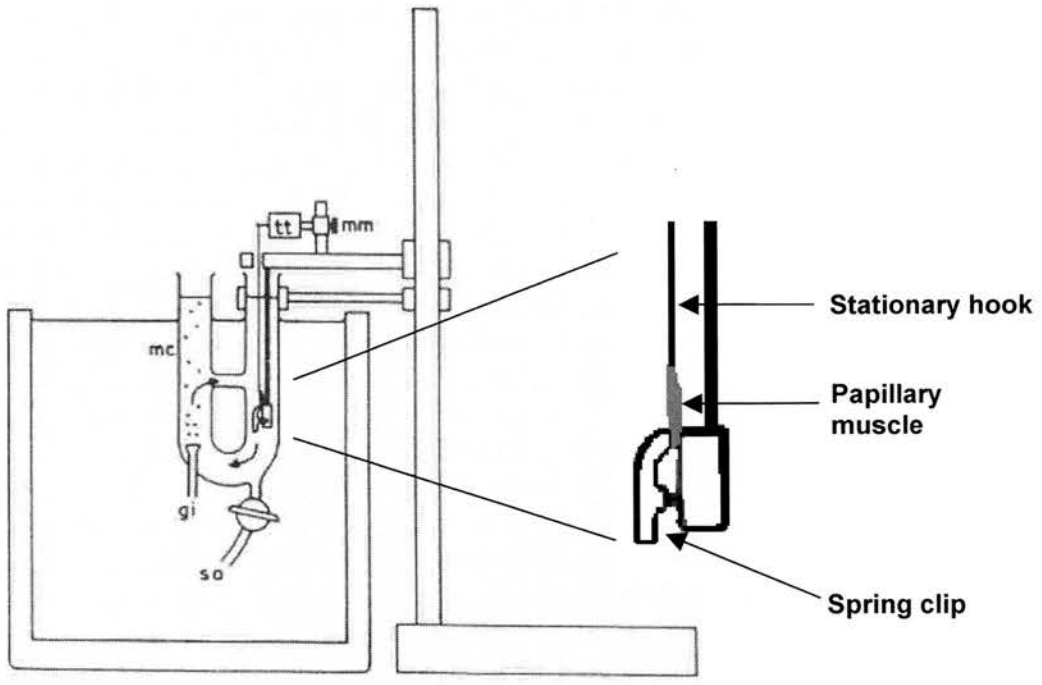


Figure 2.4. Apparatus for stimulating and recording tension from vertically mounted, isolated papillary muscles. tt, tension transducer; mm, micromanipulator; mc, muscle chamber; gi, gas inlet; so, solution outlet. Illustration modified from (Allen, 1983).

2.2.3 Calculations & data normalisation

The following contractile parameters were analysed: developed tension (active tension produced by the muscle when stimulated, mN/mm^2), time to peak tension (TPT, time from the beginning of the contraction to peak tension in ms), and time to 50% relaxation (RT_{50}, time from peak tension to 50% of relaxation in ms) (see *Figure 2.5*).

Cross-sectional area (CSA) was determined as a ratio of blotted muscle weight (W) to length (ML) by assuming a density of 1.06 gcm^{-3} (CSA=W/ML x 1.06, Hill, 1931).

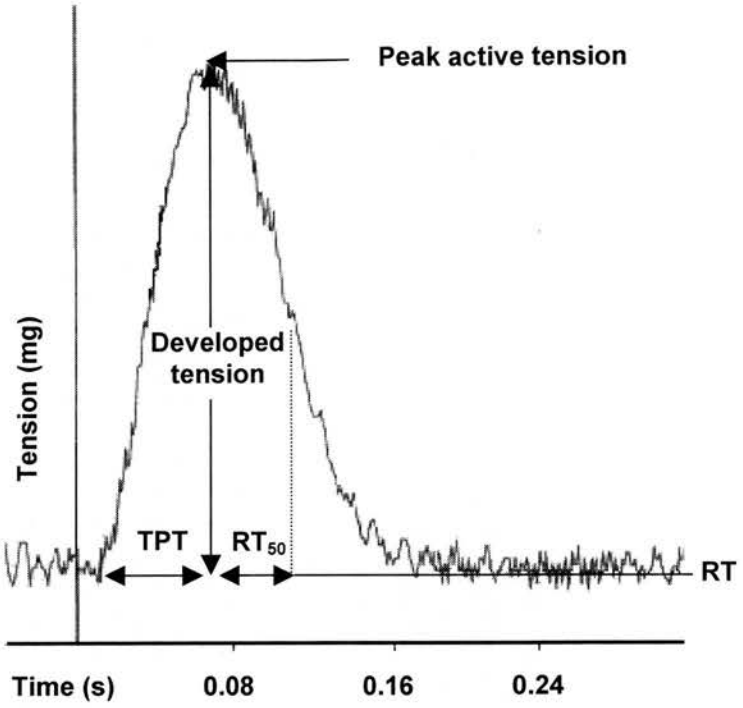


Figure 2.5. Illustration of a single contractile twitch indicating the contractile parameters measured. Developed tension, peak active tension; TPT, time to peak tension; RT_{50}, time to 50% relaxation; RT, resting tension.

2.2.4 Investigation of collagen content in papillary muscles from CAL and sham-operated rats

Following the functional investigation, papillary muscles were fixed in formalin and processed as described above (see Section 2.1.3.2.3). Histological sections (3μm thick) were prepared and stained by van Gieson's method for collagen as previously described. Slides were viewed under a microscope (Axioskop, Zeiss, Leica, Germany) attached to a digital camera (ProgRes 3012, Kontron Elektronik). An independent observer (Mr. Mark Patrizio), unaware of surgical outcome, assessed the sections by assigning a score (1-4, where 1 is negligible and 4 is abundant) to the collagen content of each papillary.

2.3 Semi-Quantitative Reverse Transcriptase Polymerase Chain Reaction

2.3.1 Introduction

Reverse transcription polymerase chain reaction (RT-PCR) is the most sensitive method for the detection of low-abundance mRNA, often obtained from limited tissue samples. It allows reproducible quantification of gene expression and has the ability to identify altered patterns of gene expression in different sample populations. Several RT-PCR strategies are available for the quantification of mRNA such as, quantitative and semi-quantitative methods. Quantitative procedures include non-competitive, or more commonly, competitive RT-PCR techniques, which depend on spiking the RNA samples before the RT step with known amounts of RT-PCR-amplifiable competitors. This section describes the development of a semi-quantitative RT-PCR approach involving an external control, the ubiquitously expressed housekeeping gene glyceraldehyde-3-phosphate (GAPDH) and a serial dilution method for verification. The approach was used to quantify (i) the ET system in failing and nonfailing rat myocardium and (ii) transmural expression of cytoskeletal proteins, the ET system and a hypertrophic marker in human failing myocardium from explanted hearts.

All RT-PCR methods assume equal reaction efficiencies, therefore, if reactions are kept within the exponential range a linear relationship will exist between the amount of input template and the amount of amplification product.

2.3.2 General laboratory practice

All work was performed using RNase-free techniques to protect samples from RNases present in the environment. Gloves were worn at all times and work surfaces and equipment were regularly cleaned with RnaseZap (Ambion). All solutions were made using a 0.1% solution of the RNase inhibitor diethyl pyrocarbonate (DEPC) in distilled water followed by autoclaving (see section 2.4). At all times during processing, samples were kept on ice to slow any degradation.

2.3.3 Isolation of mRNA

(i) Rat tissue

Total mRNA was isolated from rat myocardium using a modified version of the acid guanidinium thiocyanate-phenol-chloroform method previously described by Chomczynski & Sacchi (1987). Each sample (~50mg) was removed from the freezer and immersed in 1ml of denaturing solution (solution D, see Solutions section 2.4) where it was minced on ice and homogenised at room temperature. An equal volume of water-saturated phenol (Fisher Scientific, Loughborough, U.K.) was then added, followed by 50µl of 2M sodium acetate (pH 4). The homogenate was mixed thoroughly by inversion, followed by the addition of 100µl of chloroform-isoamyl alcohol (25:1). This final suspension was shaken vigorously for 15 seconds and left on ice for 15 minutes. Subsequently, the samples were centrifuged at 10,000*g* (Biofuge A centrifuge rotor) for 15 minutes at 4°C and the upper aqueous phase, containing the RNA, was pipetted off into a fresh tube. Care was taken not to disturb the interphase and phenol phase where DNA and protein subsist. An equal volume of isopropanol (500-600µl) was added to the lysate and the contents were mixed by inversion before being placed at -20°C for at least 1 hour. Following RNA precipitation, the samples were centrifuged (10,000*g*) at 4°C for 10 minutes, the supernatant was removed and the resulting pellet washed twice in 1ml of 75% ethanol. The pellets were then dried at room temperature, dissolved in 30µl of DEPC H_2O and stored at -20°C.

All hazardous waste reagents (phenol, guanidinium thiocyanate solutions etc.) were disposed of according to University policy of waste management.

(ii) Human tissue

Sample tissue was acquired from explanted hearts of patients undergoing transplant surgery at the Scottish Cardiac Transplant unit, Glasgow. LV epicardial and endocardial sections were dissected out, snap frozen in liquid nitrogen and stored at -70°C.

Total mRNA was isolated from frozen human myocardial samples with the use of QIAGEN RNeasy® midipreps (QIAGEN Ltd, Crawley, U.K), as outlined by the manufacturer. Briefly, 60-80mg of tissue was disrupted and homogenized in 2ml of QIAGEN lysis buffer RLT, a reagent containing β-mercaptoethanol and a highly denaturing guanidine isothiocyanate salt. A digestion step was incorporated by adding 4mls of double-distilled water along with 65µl proteinase K solution (>600 mAU/ml, QIAGEN) and incubated at 55°C for 20 min. The homogenate was then centrifuged for 5 min at 4000*g* (Model J-6B centrifuge, Beckman Coulter U.K. Ltd. High Wycombe, U.K.) between 20-25°C and the supernatant transferred into a fresh tube. 0.5 volumes of ethanol was added and mixed well by pipetting. Subsequently the lysate was passed through a QIAGEN RNeasy spin column (silica-gel-based membrane) in 3ml aliquots, aided by centrifugation (same conditions as before). All flow-through was discarded and 4ml of buffer RW1 (a reagent containing guanidine isothiocyanate) was added to the column. Following a centrifugation step (see conditions above), 2.5ml of RPE (ethanol) buffer was applied to wash the bound RNA and spun for 2 min at 4000*g* (20-25°C). Subsequently, another 2.5ml of RPE buffer was added to the spin column and centrifuged for 5 min (conditions as above) to dry the RNeasy membrane. RNA was recaptured from the spin-column membrane into a fresh tube by incubating with 100µl RNase-free water for one minute before spinning at 4000*g* for 3 min, 20-25°C. To increased the yield, the elute was passed through the membrane a second time and respun. Samples were then stored at -20°C until use.

2.3.4 RNA integrity and quantification

2.3.4.1 Agarose gel electrophoresis

Agarose gel electrophoresis was used to check the integrity of the RNA yield. An agarose gel (0.8%; LE Agarose, Seakem, BMA, Rockland, U.S.A.) was made with tris-boric acid (TBE) buffer (see Solutions section 2.4) and (0.04%) ethidium bromide (500µg/µl, Sigma, Dorset U.K.). The gel was placed in an electrophoresis tank (Sub-cell, Model 96, Bio-Rad Laboratories Ltd, Hemel Hempstead, U.K.) and

immersed in TBE buffer. Wells were loaded with 3µl of sample and 4µl of bromophenol blue loading solution (Promega, Southampton, U.K.). Subsequently, the gel was run (100V) until the loading dye had migrated two thirds of the way down the gel. The RNA was visualised in an ultraviolet transilluminator (Ultra-violet Products Ltd., Cambridge, U.K.) attached to a video display unit (SONY Corporation, Japan). Good quality RNA samples showed clearly defined bands corresponding to 28S, 18S and 5S ribosomal RNA. Examples of good quality RNA are demonstrated in *Figure 2.6*.

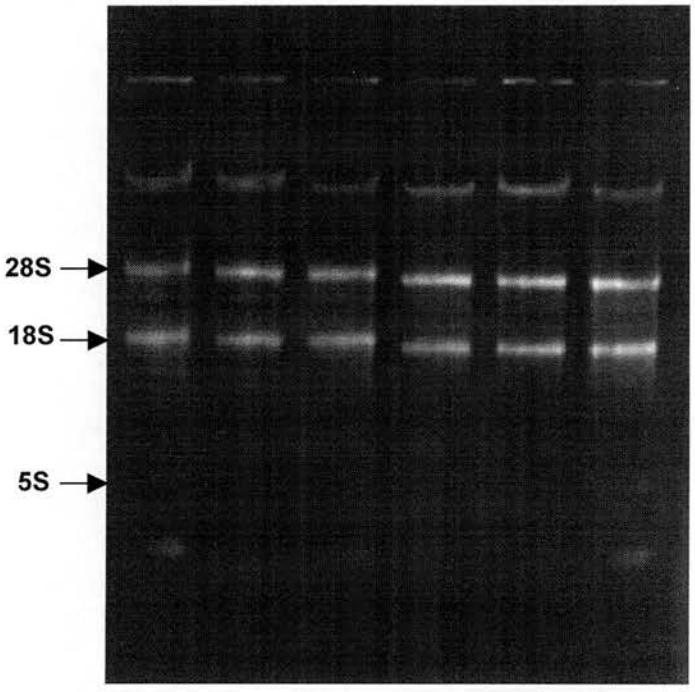


Figure 2.6. An image of an electrophoresed agarose gel representative of good quality RNA demonstrating clearly defined bands at S28, S18 and S5.

2.3.4.2 RNA quantification

The concentration and purity of total RNA was determined by measuring the absorbance at 260nM (A_{260}) and the ratio of A_{260}/A_{280} respectively in a spectrophotometer (Bio-Rad SmartSpec 3000, Bio-Rad, U.K.). The absorbance was zeroed using DEPC H_2O. RNA samples were diluted 100 fold with DEPC H_2O and placed in a quartz cuvette (200µl, UV transparent cuvette, Bio-Rad, U.K.). The spectrophotometer simultaneously measured at wavelengths of 260 and 280nM, and

calculated the concentration and the ratio of A_{260}/A_{280}. Values of total RNA yield ($\mu g \cdot \mu l^{-1}$) were obtained by adjusting for the dilution factor. A ratio of A_{260}/A_{280} in the range of 1.8 to 2.1 was taken as an indication of pure RNA. For spectrophotometers that do not calculate the value, an absorbance of 1 unit at 260nM corresponds to 40μg of RNA per ml ($A_{260} = 1 = 40\mu g \cdot ml^{-1}$).

Where RNA concentration was below $0.2\mu g \cdot \mu l^{-1}$, an ethanol precipitation step was carried out. The RNA was precipitated by adding 2.5 volumes of absolute ethanol (stored in freezer) and 0.1 volume of 3M sodium acetate (pH5.2). Samples were placed at -20°C for a minimum of 20 minutes before centrifugation at 12000*g* for 15 minutes at 4°C (Biofuge A centrifuge). The supernatant was carefully removed and 200μl of 70% ethanol was added. Subsequently, samples were centrifuged at 12000*g* for 10 minutes at 4°C. The supernatant was then removed and the RNA pellets left to air-dry. Pellets were then resuspended in 20μl or 60μl DEPC H_2O (rat and human respectively) and the concentration reanalysed using the spectrophotometer (BIO-RAD SmartSpec 3000, Bio-Rad, U.K).

2.3.5 RT-PCR

2.3.5.1 Synthesis of first strand cDNA

Total RNA (2μg) was primed with 100pM oligo-dT_{15} and reverse-transcribed in the presence of 200U Moloney-murine leukemia virus reverse transcriptase (M-MLV reverse transcriptase; Promega), 0.5mM dNTPs and first strand buffer (final concentration; 50mM Tris-HCL, pH 8.3 at 37°C; 75mM KCl; 3mM $MgCl_2$) in 20μl. The incubation was performed in a PCR thermocycler (Primus 25/96 Thermocycler, MWG-Biotech, Milton Keynes, U.K for rat and PTC-200 Peltier thermal cycler, MJ Research Inc., Waterown, U.S.A. for human) at 37°C for 45 minutes, then at 95°C for 5 minutes to denature the reverse transcriptase. Oligo-dT_{15} was purchased from MWG-Biotech, all other reagents were purchased from Promega.

2.3.5.2 Primer design

Primers were designed to span introns to avoid false positive results arising from amplification of contaminating genomic DNA.

(i) Rat tissue

Gene-specific primers were designed for ppET-1, ET_A, ET_B and ANP using Genejockey II software (Biosoft, Cambridge, U.K.) and were synthesised by MWG-Biotech according to previously published oligonucleotide sequences. The primer sequences and their sizes are summarised in *Table 2.1*. For semi-quantitative analysis, the constitutively expressed enzyme GAPDH served as an external control in the calculation of the densitometric results.

(ii) Human tissue

Published cDNA sequences for human GAPDH, BNP, ppET-1, ET_A, ET_B, gelsolin and titin were obtained and primers were designed using Genejockey II software. Isoform specific primers (N2A and N2B) were designed within the unique conserve sequences for each isoform (Freiburg *et al.*, 2000). All primer sequences and their respective product sizes are shown in *Table 2.1*.

2.3.5.3 Primer optimisation

Optimal PCR conditions were determined methodically for each primer pair. Annealing temperature was estimated from the GC content and tested over a range (2°C increments), while the effect of $MgCl_2$ concentration on reaction efficiency was investigated between 1.75 - 2.50mM. The desired annealing temperature and $MgCl_2$ concentration were those which produced the greatest amount of desired product with the least amount of extranous product (see *Figure 2.7*). Subsequently, PCR was performed to the point of plateau. The reaction was paused every 2 cycles (after 16) and a (5%) sample removed. Products were visualised on a 1% agarose gel and a cycle number was chosen within the exponential range of amplification to provide a linear relationship between the amount of input template and the amount of

amplification products for each primer pair (see *Figure 2.8*). For rat and human primer profiles, see *Table 2.1*).

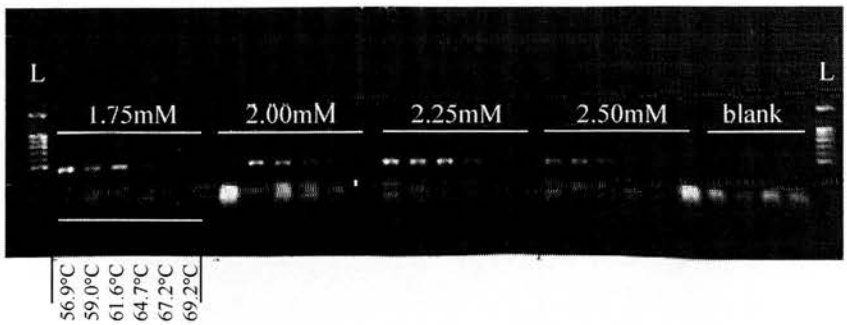


Figure 2.7. A representative scan of an electrophoresed agarose gel, demonstrating optimisation of human GAPDH primers. Optimum yield of desired product observed at an annealing temperature of 57 ℃ and a [Mg^{2+}] of 1.75mM. L, 50 base pair DNA ladder. Temperature range 56.9, 59.0, 61.6, 64.7, 67.2, 69.2 ℃ increments for each concentration of Mg^{2+}.

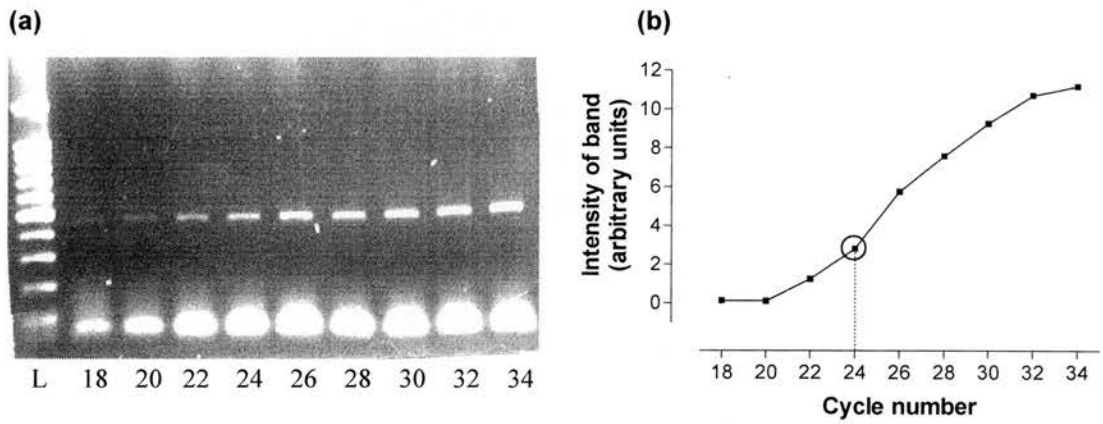


Figure 2.8. Agarose gel (a) and associated graph (b) of PCR cycle number: product relationship. L, 50 base pair DNA ladder.

2.3.5.4 PCR

(i) Rat tissue

Ten microlitres of the cDNA from the RT reaction was used for PCR amplification. Each PCR reaction (50µl) contained 2.5U *Taq* DNA polymerase (Promega), 200µM dNTPs (Promega), 100pM specific primers for gene of interest (MWG-Biotech), 10x PCR reaction buffer (final concentration, 10mM Tris-HCl, pH 9.0, at 25°C, 50mM KCl and 0.1% Triton, Promega) and $MgCl_2$ (Promega) at a concentration specific to each primer (see Table 2.1). The samples were transferred directly from ice into the thermocycler (MWG-Biotech) and the DNA was denatured for 4 minutes at 94°C. Subsequently, *n* cycles (see *Table 2.1*) of amplification were performed, denaturation (1 min at 94°C), annealing (1 min at a temperature specific to each primer, see *Table 2.1*) and elongation (2 mins at 72°C). After the last cycle the 72°C elongation step was extended to 10 mins. Negative control tubes (no cDNA) with DEPC H_2O were included to ensure the reactions were not contaminated.

(ii) Human tissue

Five microlitres for human cDNA was used in a 25µl total reaction volume PCR and amplified in a PTC-200 Peltier thermal cycler (MJ Research Inc.). The PCR mixture was made up as before and samples were amplified under the conditions in *Table 2.1.*

2.3.5.5 Verification

Verification of the alterations in mRNA levels in LV epi- and endocardial samples from end-stage heart failure patients was carried out. A two-fold serial dilution of each cDNA sample was made and used as template for PCRs that were carried out on each primer pair.

2.3.6 Determination of the absolute alterations of mRNA

2.3.6.1 Agarose gel electrophoresis and densitometry

The PCR products were separated on an agarose gel (1%; Seakem) and stained with ethidium bromide (0.04%). A 100 base-pair stepladder (Promega) was used for products to be identified by their size. The expression of the PCR product was quantified by densitometry (FLA2000, Fujifilm, Fuji Photo Film Co., Ltd, Tokyo, Japan; AIDA 2D Densitometry software 2.11, raytest Isotopenmessgeraete GmbH, Straubenhardt, Germany). Changes in transcription of the genes investigated were determined by comparison of gene-of-interest/GAPDH ratios in rat LV, RV and septum and between human epicardial and endocardial samples and dilution curves were plotted to confirm the changes of expression.

2.3.6.2 Sequencing

The specificity of the amplified sequences was confirmed by DNA sequencing. The PCR product was cut from the agarose gel and purified using a GFX purification kit (Amersham Pharmacia Biotech UK Limited, Buckinghamshire, U.K.). Briefly, the DNA of interest was specifically bound to a glass fibre matrix using a chaotropic agent that denatures protein and dissolves agarose gel. The column was centrifuged briefly following incubation at 60°C to allow the agarose to dissolve completely. The fibre matrix containing the DNA was then rinsed with wash buffer (Tris-EDTA buffer with absolute ethanol to a final concentration of 80%) and spun briefly at full speed (30 seconds; 16,000*g*) before the purified DNA was reclaimed from the fibre matrix with 30µl double distilled autoclaved H_2O. The DNA was precipitated as previously described for RNA (see section 2.3.4.2) and recovered (dry) samples were sent to MWG-Biotech for sequencing.

Table 2.1.PCR primer sequences and profiles for rat and human genes

Sequence	GeneBank Accession No.	Product Size (bp)	Annealing Temp. (°C)	Number of cycles	[$MgCl_2$]
Rat GAPDH 5'-gccatcaacgacccttcatt-3' 5'-tgccagtgagcttcccgttc-3'	M17701	597	55	22	1.75
Rat ANP 5'-atgggctccttctccatcacc-3' 5'-tccgctctgggctccaatcctgt-3'	M27498	426	57	28	2.00
Rat preproET-1 5'-tcttctctctgctgtttgtg-3' 5'-tagttttcttccctccacc-3'	M64711	481	58	29	1.75
Rat ET_A receptor 5'-atcgctgacaatgctgagag-3' 5'-ccacgatgaaaatggtacag-3'	M60786	225	52	32	2.25
Rat ET_B receptor 5'-caaaatggacagcagtagaaa-3' 5'-gacttaaagcagtttttgaatct-3'	NM017333	552	58	28	2.00
Human GAPDH 5'-accacagtccatgccatcactg-3' 5'-gtccaccaccctgttgctgtag-3'	M33197	451	57	24	2.25
Human BNP 5'-gttcagcctcggacttggaa-3' 5'-gctcaaaggtaagaaaccatct-3'	M25296	455	51	24	2.25
Human preproET-1 5'-ccaaggagctccagaaacagc-3' 5'-gcacattggcatctattctca-3'	NM001955	290	47	32	2.50
Human ET_A 5'-cttggagaccttatctatgtgg-3' 5'-gggcatacagaaatagaacccg-3'	L06622	431	57.6	29	2.25
Human ET_B 5'-ttggagctgagatgtgtaagctg-3' 5'-ttcagtgaagccatgttgatacc-3'	L06623	627	57.6	29	2.25
Human gelsolin 5'-ggctgttgaggtattgcc-3' 5'-tcaccaggaacctcttcg-3'	X04412	329	51	28	1.75
Human Titin 2A 5'-ggacaaaagggaagtggagacagc-3' 5'-gtgtatgtcggtcaccatcaatgc-3'	X90569	680	63.8	24	1.75
Human Titin 2B 5'-tcaagccatctcggttctttagaatgactc-3' 5'-gttcggcgctatgaagtccttcttcctcgg-3'	X90568	263	63	28	1.75

2.4 Solutions, drugs and chemicals

Salts were purchased from Sigma-Aldrich Company Ltd., Dorset, U.K., unless otherwise stated.

Saline
0.9w/v sodium chloride solution.

Heparin
Heparin Sulphate 5000 Uml^{-1}, Multiparin, CP Pharmaceuticals, Wrexham, U.K. diluted to 100 $U \cdot ml^{-1}$ in saline.

Tyrode's
Tyrode's solution (pH 7.4) contained (mM): 118.0 NaCl, 5.0 KCl, 1.0 $MgSO_4.7H_2O$, 20.0 $NaHCO_3$, 1.0 NaH_2PO_4, 2.0 $CaCl_2$ and 10.0 glucose.

A x10 stock was prepared without $CaCl_2$ or glucose and stored in the fridge. On experimental day, the stock was diluted and $CaCl_2$ and glucose were added fresh.

Tyrode's/BDM
The cardioplegic solution contained Tyrode's and 30.0mM, 2,3-butanedione-monoxime (BDM) this was also freshly prepared each day.

Tespa Coating
Slides were immersed for 10 seconds in each of the following:

1. 10% Hydrochloric Acid in 70% ethanol
2. Sterile water
3. 100 Acetone

The slides were air dried and then placed for 10 seconds in each of the following:

4. 2% Aminopropylethoxysilane (TESPA) in Acetone
5. 100% Acetone
6. 100% Acetone

Slides were air dried again and were stored for one month in an air tight container.

Van Gieson's stain for collagen
Saturated aqueous picric acid 100mls
1% acid fuchsin 10mls

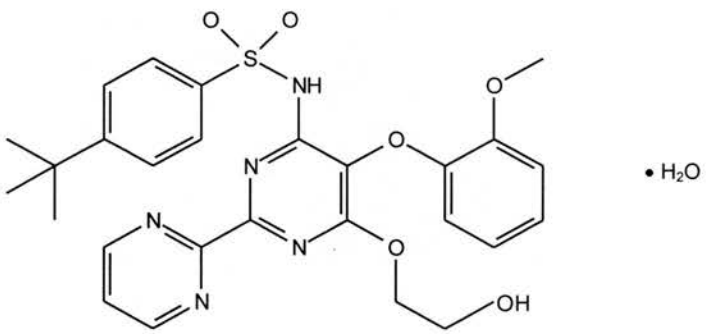
DEPC H_2O
5 drops of diethyl pyrocarbonate (DEPC) were added to 500mls of distilled H_2O. The bottle was shaken vigorously for 30 seconds and left to stand for 20 minutes. The solution was then shaken again and left for 1 hour before being autoclaved.

Solution D

Solution D (pH 7.0) contained 4M guanidinium thiocyanate, 25mM sodium citrate (pH 7) and 0.5% sarcosyl. 360µl β-mercaptoethanol was added/ 50ml of solution. This solution was kept in a dark bottle at room temperature for a month.

TBE

TBE contained 0.45M Tris base, 0.445M Boric acid and 0.5M EDTA (pH 8.0) made up to 1L with sterile water.



Ro 47-0203/029

Bosentan (Ro 47-0203/029)

Dr. Martine Clozel (Actelion Pharmaceuticals Ltd., Basel, Switzerland), generously supplied Bosentan (Ro 47-0203/029).

Chronic bosentan administration

A 5% gum arabic solution was prepared and stirred for 1 hour before being filtered. Bosentan was then added (100mg/4ml) and a polytron was used (30 sec) to make a microsuspension. This was administered to the rat via gastric gavage (100mg·kg^{-1}).

Acute bosentan administration

Bosentan (30 mg·kg^{-1}) was dissolved in 5% DMSO and infused intravenously.

Chapter 3

Comparison of the expression and function of the endothelin system in failing and non-failing rat myocardium

3.1 Introduction

Endothelin-1 (ET-1) is a powerful mediator of vasoconstriction, however, its exact role as a regulator of myocardial contractile function is unclear. Direct positive inotropic effects of ET-1 have been demonstrated in some preparations but responses are complex and tend to vary depending on the preparation used, experimental conditions and the species involved. Various studies provide evidence that ET-1 exerts positive inotropic responses in isolated cardiomyocytes (Kelly *et al.,* 1990; Kramer *et al.*, 1991) and isolated cardiac tissue preparations from animals (Li *et al.*, 1991) and humans (Meyer *et al.,* 1996). Controversially, positive inotropic responses have not been detected in isolated whole heart preparations from rats (Neubauer *et al.*, 1990), rabbits (Karwatowska-Prokopczuk & Wennmalm, 1990) or *in vivo* studies (Yang *et al.*, 1991; Beyer *et al.*, 1994), possibly due to ischaemia resulting from coronary vasoconstriction.

The cellular basis for the actions of ET-1 on myocardial tissue is highly complex and still not fully understood. Studies have shown that both ET receptors can couple to at least two distinct signal transduction pathways leading to adenylyl cyclase inhibition and formation of inositol-1,4,5-triphosphate (IP_3) and 1,2-diacyl glycerol (DAG), via stimulation of phospholipase C (PLC) (Vogelsang *et al.*, 1994). Activation induces a rise in cytosolic Ca^{2+} and sensitises cardiac myofilaments to intracellular calcium (Kramer *et al.*, 1991), thus increasing the force of contraction. However, it is still unclear as to which receptor(s) is responsible for modulation of myocardial contractility. Many factors are responsible for stimulating synthesis and release of ET-1 including stretch stimulated release of ET-1. Previous studies have shown that cultured endothelial cells (Kuchan & Frangos, 1993; Macarthur *et al.*, 1994) and cardiomyocytes (van Wamel *et al.*, 2001) release ET-1 upon mechanical stretch.

In an attempt to maintain cardiac output and peripheral perfusion, many compensatory mechanisms are activated in response to myocardial infarction (MI). Cardiac upregulation of prepro ET-1 (ppET-1) mRNA has been reported and a significant elevation of plasma ET-1 and big ET-1 concentration is also observed, correlating positively with disease severity during progression from MI to chronic

heart failure (CHF) (Tønnessen *et al.*, 1997). However, ET receptor mRNA levels do not diminish following MI, as is the case with adrenergic receptors in response to increased catecholamine levels. Cardiac upregulation of both ET_A and ET_B receptors has been demonstrated in the rat model of experimental heart failure at 3 (Sakai *et al.*, 1996a), 4 (Picard *et al.*, 1998) and 12 weeks (Sakai *et al.*, 1996b) post MI. However, studies in our laboratory, using a model of less severe heart failure, have shown that ET_B receptor mRNA is selectively increased in the LV during the period from 5-12 weeks post coronary artery ligation (CAL) (Sherry, 2000; Smith *et al.*, 2000), suggesting that disease severity is important in regulation of the ET system.

Administration of ET receptor antagonists has been shown to exert favourable haemodynamic effects, improve cardiac function and prevent ventricular remodelling in the rat model of CHF (Teerlink *et al.*, 1994; Sakai *et al.*, 1996a, 1996b). This highlights the importance of the ET system during experimental HF and suggests that increased expression of ET-1 and ET receptors in the failing ventricle may contribute to cardiac remodelling. In addition, raised levels of endogenous ET-1 may directly influence cardiac contractility in pathological conditions or contribute to the Frank-Starling response by increasing peripheral resistance.

The aims of this study were to evaluate the effects of exogenous ET-1 on cardiac contractility in the rat CHF model by comparing the contractile behaviour of papillary muscles from sham and CAL rats and to determine whether increased ET_B receptor expression influenced the response. In addition, the relative changes in the density of ET_A and ET_B receptor subtypes were established.

3.2 Methods

Initial procedures for the induction and characterisation of MI and the preparation of papillary muscles were carried out as described in Chapter 2. After complete mechanical stabilisation (section 2.2.2), a cumulative concentration-response curve to isoprenaline (10^{-9}-10^{-6} M) was established. Following washout and return to a steady state, the concentration of ET-1 in the organ bath was increased in a cumulative manner in steps of 0.5 log units (10^{-11} to 10^{-7} M). When steady contractile

force had been achieved, ET-1 was added to yield the next higher concentration. Finally, after subsequent wash out and stabilisation, the preparation was challenged with $CaCl_2$ ($2x10^{-3}$ - 1.1^{-2} M). Papillary muscles were removed and measured, blotted and weighed, fixed in formalin and processed for the evaluation of collagen content (as described in section 2.2.4). All forces were normalised by cross-sectional area (CSA) of the papillary muscle. Morphological changes in heart (HW) and body weight (BW) were recorded (*Table 3.1*). The area of the infarct was measured using Image Analysis software (Carl Zeiss Vision) as described in section 2.1.3.1.3.

Subsequently, expression of the ET system and atrial natriuretic peptide (ANP), a marker of cardiac hypertrophy, were investigated in the LV, septum and RV tissue samples from sham-operated and CAL animals using semi-quantitative RT-PCR as described in section 2.3.

Comparison between groups was performed by unpaired Student's *t*-test or analysis of variance followed by Bonferroni's multiple comparisons test where appropriate. A P value <0.05 was considered significant. Correlation analysis was used to examine the interaction between the parameters measured. The Pearson correlation coefficient (r) was calculated for each simple linear regression to determine the closeness of the straight line to the points.

3.3 Results

3.3.1 Effects of coronary artery ligation on mortality

Twenty-seven rats underwent CAL for the induction of CHF. Deaths occurring during or immediately following surgery were 22% of the whole group. Most of these were attributed to post-infarct arrhythmias, a common problem associated with CAL surgery in the rat. Only one CAL animal died in the 24hr period after surgery. No early or late deaths of sham-operated animals occurred. Infarct sizes of the free LV wall measured $12.35 \pm 2.75 mm^2$ (n=5).

3.3.2 Haemodynamic measurements

At 15 weeks post-ligation, cardiac haemodynamic parameters were recorded *in vivo* under general anaesthetic using a fluid filled intraventricular catheter placed via the right carotid artery. CAL animals with LVEDPs ≥ 10mmHg were considered to have significant LV dysfunction (LVD), a significant decrease in dP/dt_{max} was also observed but no significant difference was obtained in HR, MAP or dP/dt_{min} between groups (*Table 3.1*). No significant difference was observed in BW between sham and CAL animals but the HW: BW ratio was significantly increased, despite the free wall of the infarcted area being very thin and consisting of connective tissue (*Figure 3.1*).

3.3.3 Baseline responses of isolated papillary muscles

(i) Passive length-tension relationship

In the presence of 2,3-butanedione-monoxime (BDM) to inhibit cardiac contraction, the passive length/tension relationship of rat right ventricular papillary muscle was investigated. As expected, passive stretch produced an increase in tension in papillary muscles of both groups. A greater rate of rise in tension was observed in the CAL group (*Figure 3.2*). Simple linear regression of the data, performed on a semi-log plot, indicated a significant difference in the length-tension relationship between groups ($P<0.0001$). CAL resulted in an increased slope of the straight line (*Figure 3.2*). The *y*-intercept was also increased and the condition of linearity was met (sham, $r = 0.985$; CAL, $r = 0.973$).

(ii) Active length-tension relationship

Typical changes in developed tension after muscle stretch were observed in electrically stimulated papillary muscles. It can be seen in *Figure 3.3* that twitch tension increased as the muscle was progressively stretched. Peak developed tension at L_{max} under baseline conditions was considerably greater in papillary muscles from sham animals (*Figure 3.4*). There was the tendency towards an increased resting

tension in the CAL group at L_{max} (sham, 4.59 ± 1.07mN/mm^2; CAL 9.22 ± 3.67mN/mm^2), although the difference was not statistically significant (P=0.25).

Baseline twitch timing parameters were not significantly altered between groups, time to peak tension (TPT); 53.17 ± 1.51 ms compared with 50.83 ± 2.22 ms and time from peak to 50% relaxation (RT_{50}); 41.17 ± 1.95 ms compared with 40.17 ± 1.35 ms for sham and CAL respectively.

3.3.4 Effects of β_1 agonist isoprenaline

In isolated rat papillary muscles, isoprenaline produced concentration-dependent increases in peak isometric tension and a reduction in TPT and RT_{50}, consistent with stimulation of β-adrenoceptors. Although the data were not statistically different, the pattern of responses for a range of contractile indices suggests a reduced inotropic response to isoprenaline in papillary muscles from CAL animals compared to sham animals (*Figure 3.5*).

3.3.5 Effects of ET-1 on contractile behaviour

Contractile responses to ET-1 were slow in onset, long lasting and extremely variable. In both groups ET-1 produced both positive and negative inotropic effects. It can be seen in *Figure 3.6a* that negative responses occurred at lower concentrations while more positive effects were elicited at higher concentrations (10^{-8}-10^{-7} M) in papillary muscles from sham animals. However, due to inconsistent responses, no significant difference was observed when the data were averaged. The biphasic pattern that emerged in the sham group was not as obvious in the CAL group, where the relaxation phase was less profound (*Figure 3.6b*). Linear regression analysis was used to investigate the relationship between the resting tension at L_{max} and peak response to exogenous ET-1. CAL responses produced a negative slope, indicating that a larger resting tension at L_{max}, achieved by a greater degree of stretch, tended to be associated with a reduced response to exogenous ET-1. However, the condition of linearity was not met (r = 0.212). Cumulative addition of ET-1 caused no modification of the timing parameters of contraction, TPT or RT_{50} (*Figure 3.7*).

Table 3.1. Haemodynamic and morphological characteristics of sham-operated and CAL animals indicating LV dysfunction.

	Sham (n=6)	CAL (n=6)
Body Weight (g)	430 ± 12	442 ± 15
Heart: Body Weight ratio ($g \cdot kg^{-1}$)	2.35 ± 0.09	2.81 ± 0.15*
Lung: Body Weight ratio ($g \cdot kg^{-1}$)	3.25 ± 0.09	3.23 ± 0.11
HR (bpm)	344 ± 13	332 ± 13
MAP (mmHg)	95.4 ± 4.6	99.1 ± 8.9
LVEDP (mmHg)	3.3 ± 0.2	12.2 ± 1.8**
dP/dt_{max} ($mmHg \cdot ms^{-1}$)	2606 ± 134	2045 ± 71**
dP/dt_{min} ($mmHg \cdot ms^{-1}$)	-2679 ± 170	-2294 ± 99
CSA (mm^2)	0.40 ± 0.02	0.47 ± 0.03

Values are means ± SEM, *$P<0.05$, **$P<0.01$ compared to sham. HR, heart rate; MAP, mean arterial pressure; LVEDP, left ventricular end-diastolic pressure; dP/dt_{max}, maximal rate of pressure rise; dP/dt_{min}, maximal rate of pressure decline; CSA, cross sectional area of papillary muscle.

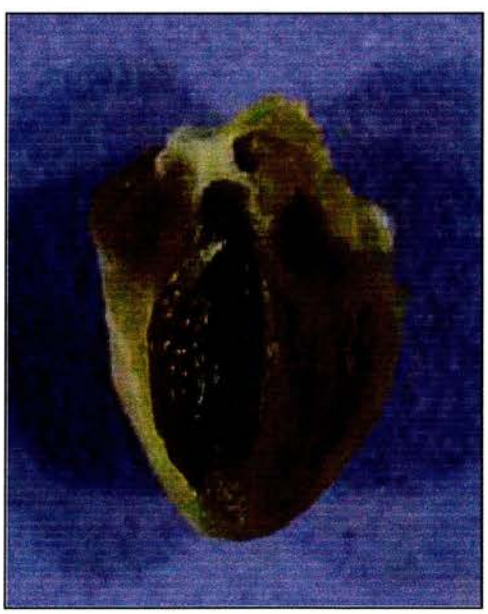


Figure 3.1. Cross section of a CAL heart cut apex to base through the infarcted region.

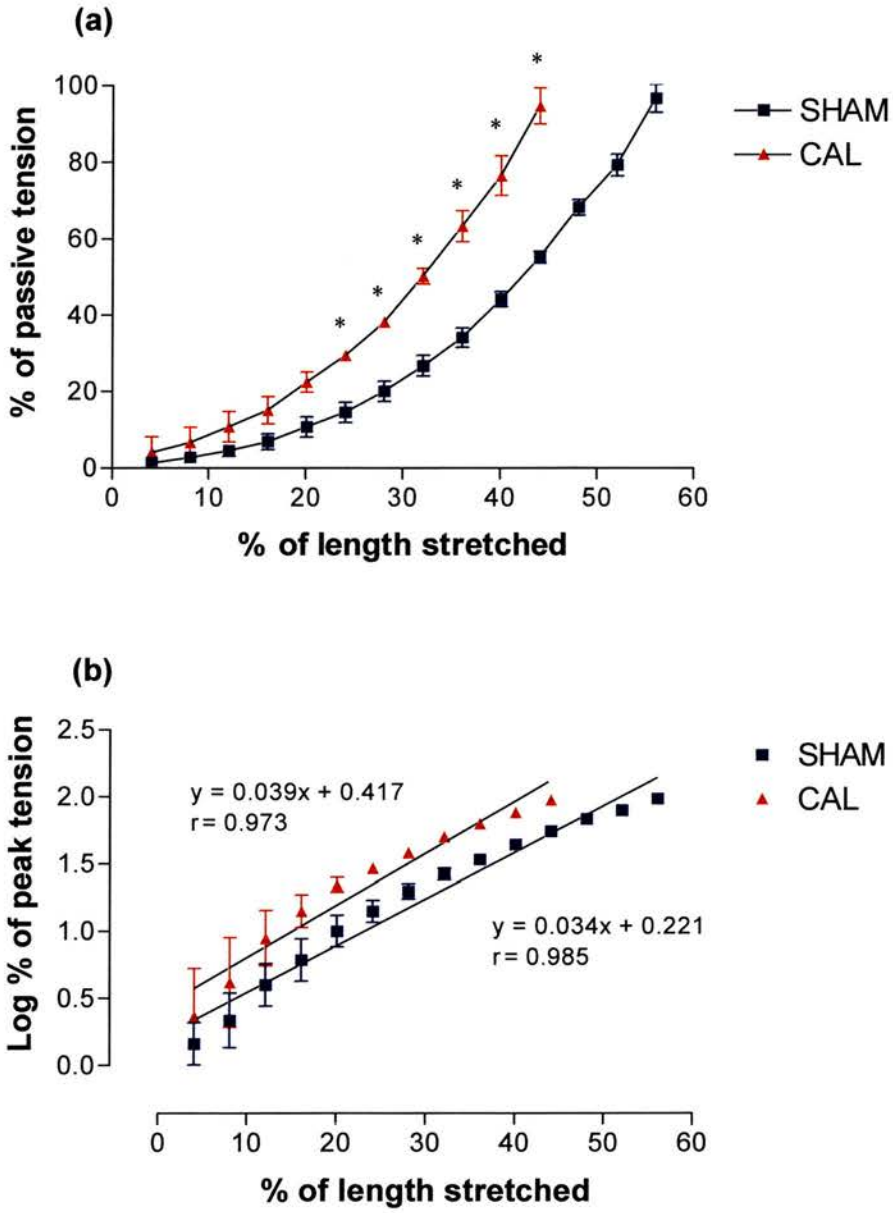


*Figure 3.2. The passive length-tension relationship of papillary muscles from sham (n=6) and CAL animals (n=6, *P<0.01) plotted from (a) basic meaned data and (b) the same data expressed on a semi-log plot, indicating linear regression analysis. The gradient of the lines and the correlation coefficient values (r) are also displayed.*

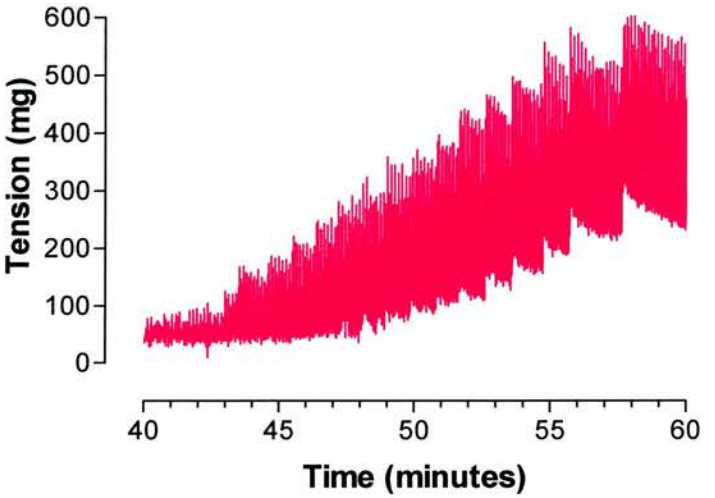


Figure 3.3. Sample trace of active twitch tension from rat right ventricular papillary muscle, progressively stretched in 0.1mm increments / minute until maximum isometric twitch tension was achieved (L_{max}).

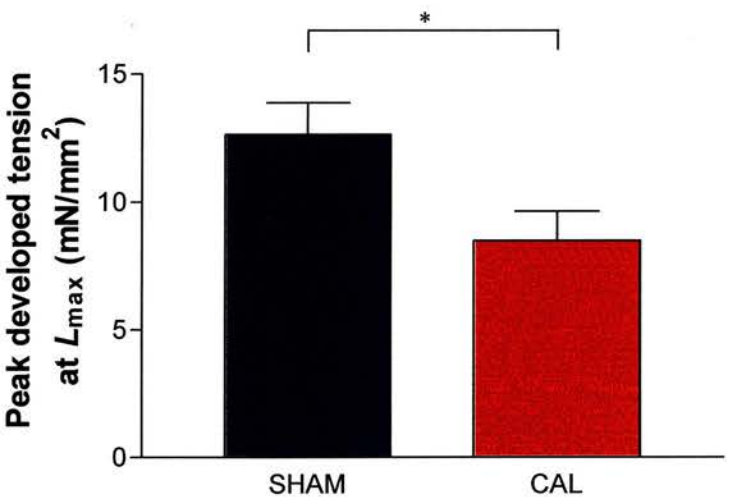


*Figure 3.4. Peak developed tension achieved at L_{max} by papillary muscles from sham (n=6) and CAL animals (n=6, *P<0.05).*

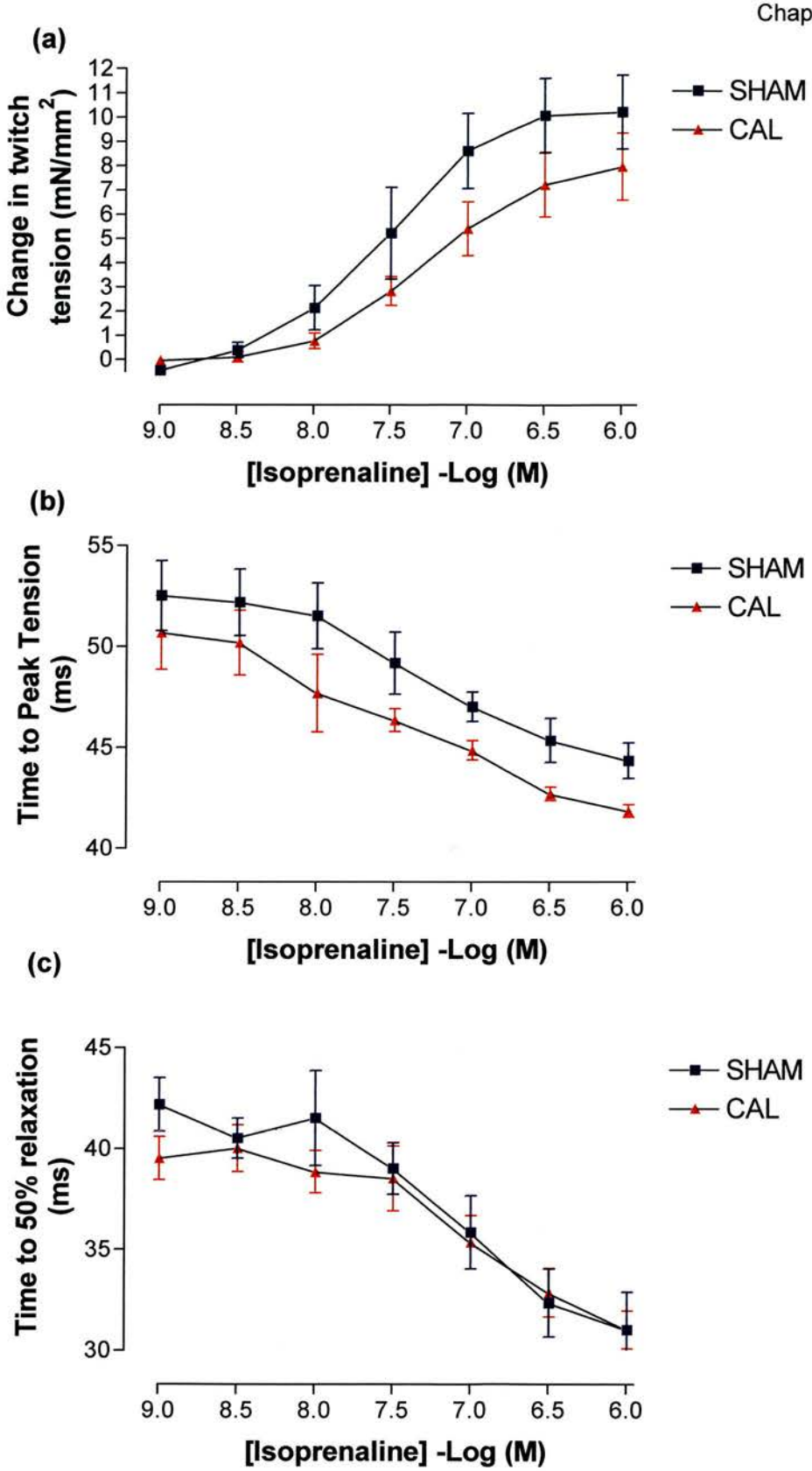


Figure 3.5. Increasing concentration of isoprenaline and its effects on (a) change in twitch tension, (b) time to peak tension and (c) time from peak to 50% relaxation of isometrically contracting papillary muscles (n=6 in each group).

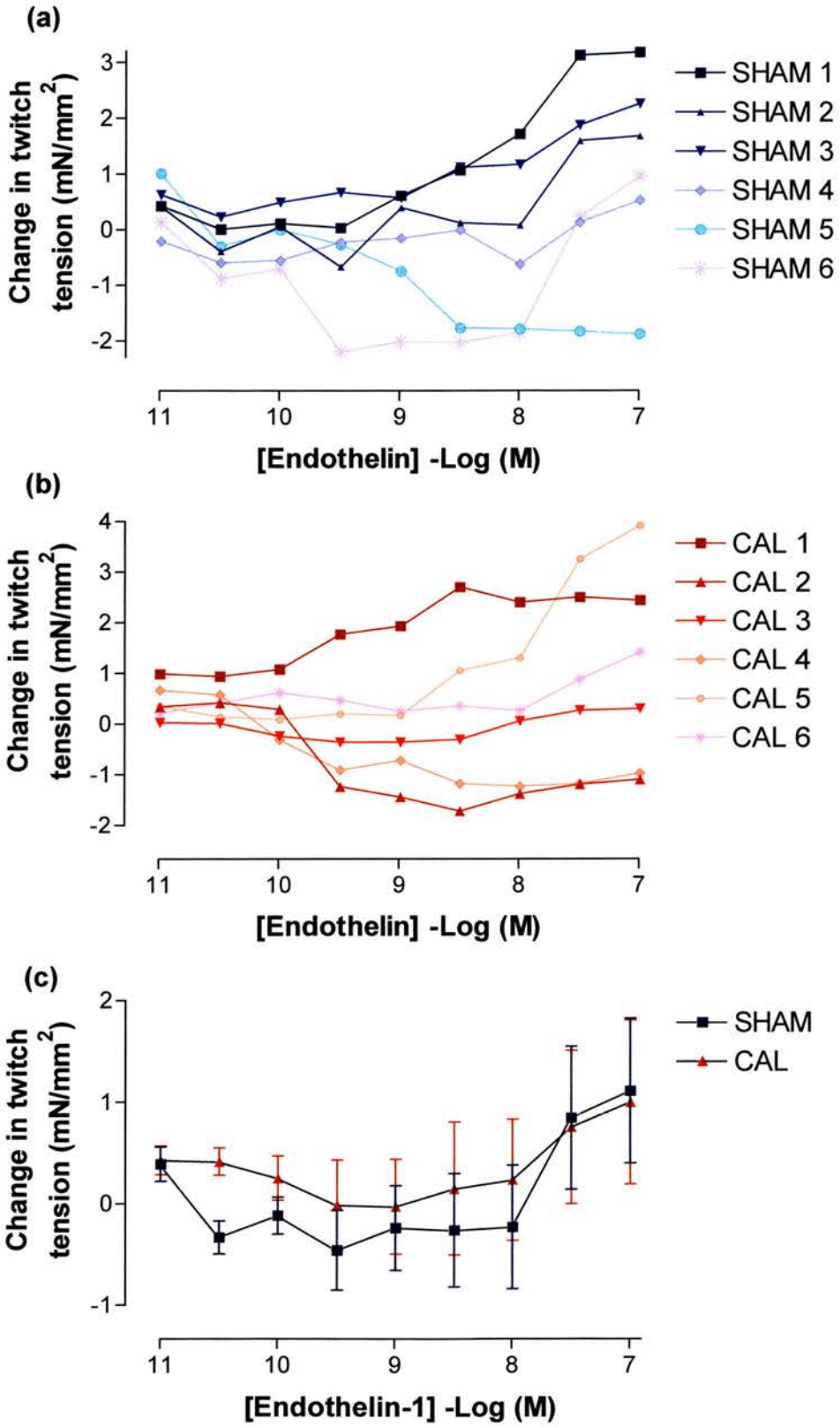


Figure 3.6. The influence of increasing ET-1 concentration on contractile behaviour of rat right papillary muscles from (a) individual sham, (b) individual CAL and (c) mean data of failing and nonfailing hearts (n=6 in each group).

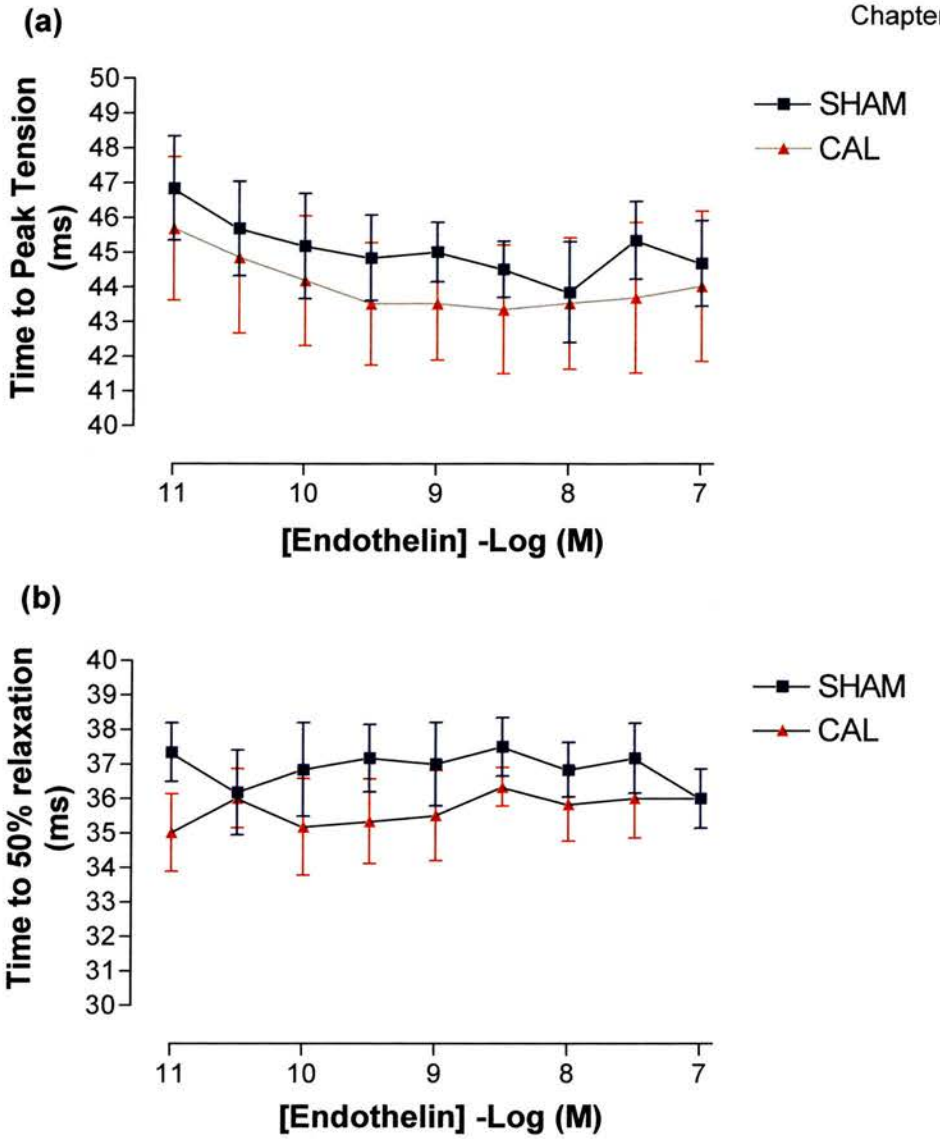


Figure 3.7. Influence of ET-1 concentration on contractile parameters (a) TPT and (b) RT_{50} of isometrically contracting papillary muscles from sham and CAL animals (n=6 in each group).

3.3.6 Influence of extracellular Ca^{2+} concentration

Addition of extracellular Ca^{2+} produced typical positive inotropic responses in stimulated papillary muscles. However, in the CAL group, a decreased responsiveness to Ca^{2+} was observed, the maximum developed tension was reduced and peak inotropic effect was evoked with 11mM compared to 9mM in the sham group (*Figure 3.8*). The positive inotropic effects of Ca^{2+} were not associated with changes in the contractile timing parameters in the sham group (*Figure 3.9*), however, although the data was not statistically significant, there was the tendency towards an increase of TPT and RT_{50} values in the CAL group.

3.3.7 Investigation of collagen content in papillary muscles from sham and CAL animals

Sections of rat right ventricular papillary muscle from sham and CAL animals were stained with van Gieson's histological stain and assessed for collagen content. Normal myocardium stained yellow, while collagen appeared pink. The nuclei were counterstained with haematoxylin (dark blue). A significant increase in collagen content was observed in the CAL papillary muscles compared to sham (*Figure 3.10*). Linear regression analysis was used to determine whether there was an association between the resting tension at L_{max} and the collagen content of papillary muscles. The slope and the y-intercept of the CAL group were greater than those for the sham group (sham, $y = 0.006x + 0.799$; CAL, $y = 0.010x$ 1.715), however, the condition of linearity was not quite met (sham, $r = 0.672$; CAL, $r = 0.829$).

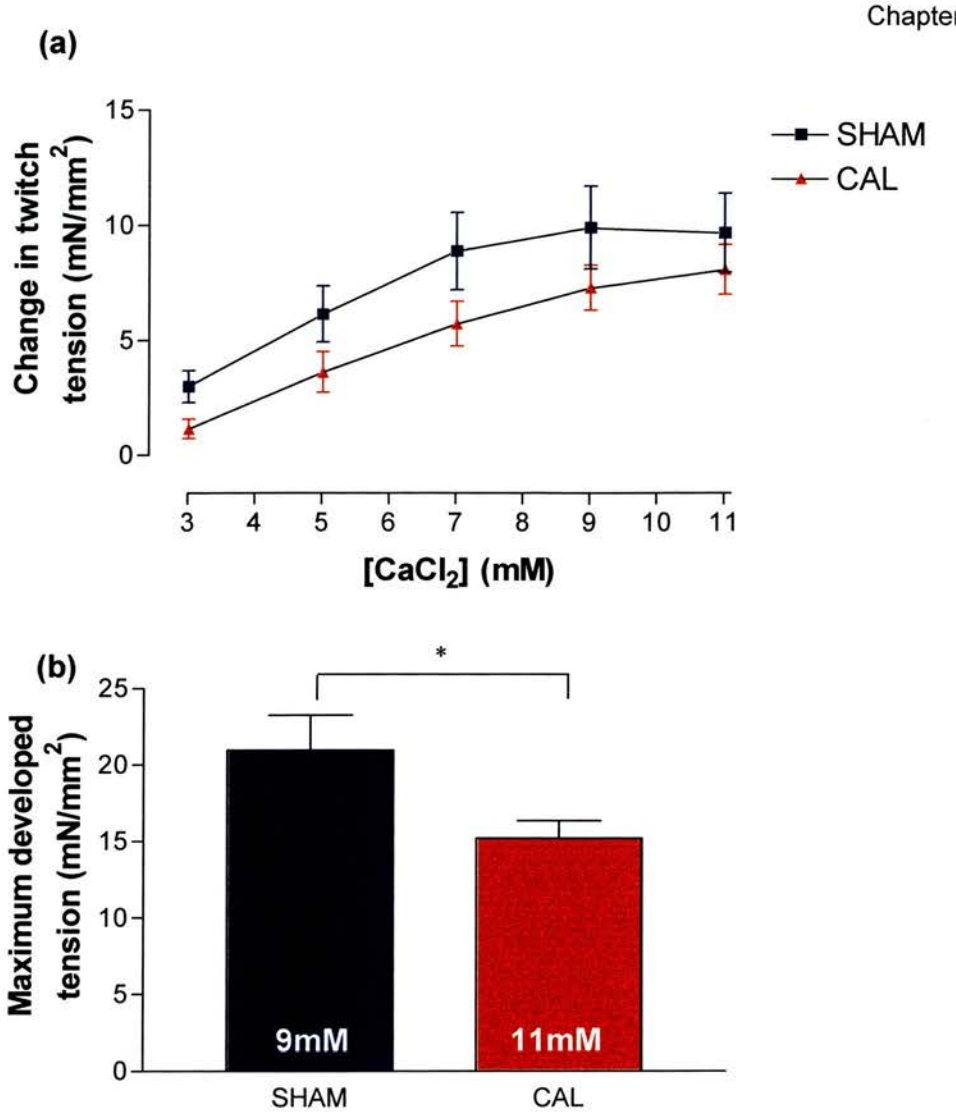


*Figure 3.8. The positive inotropic responses to extracellular Ca^{2+} concentrations in rat papillary muscle from failing and nonfailing hearts (a) developed tension in response to increasing Ca^{2+} concentration and (b) peak inotropic effect evoked by 9mM and 11mM $CaCl_2$ respectively (n=6 in each group, *P<0.05).*

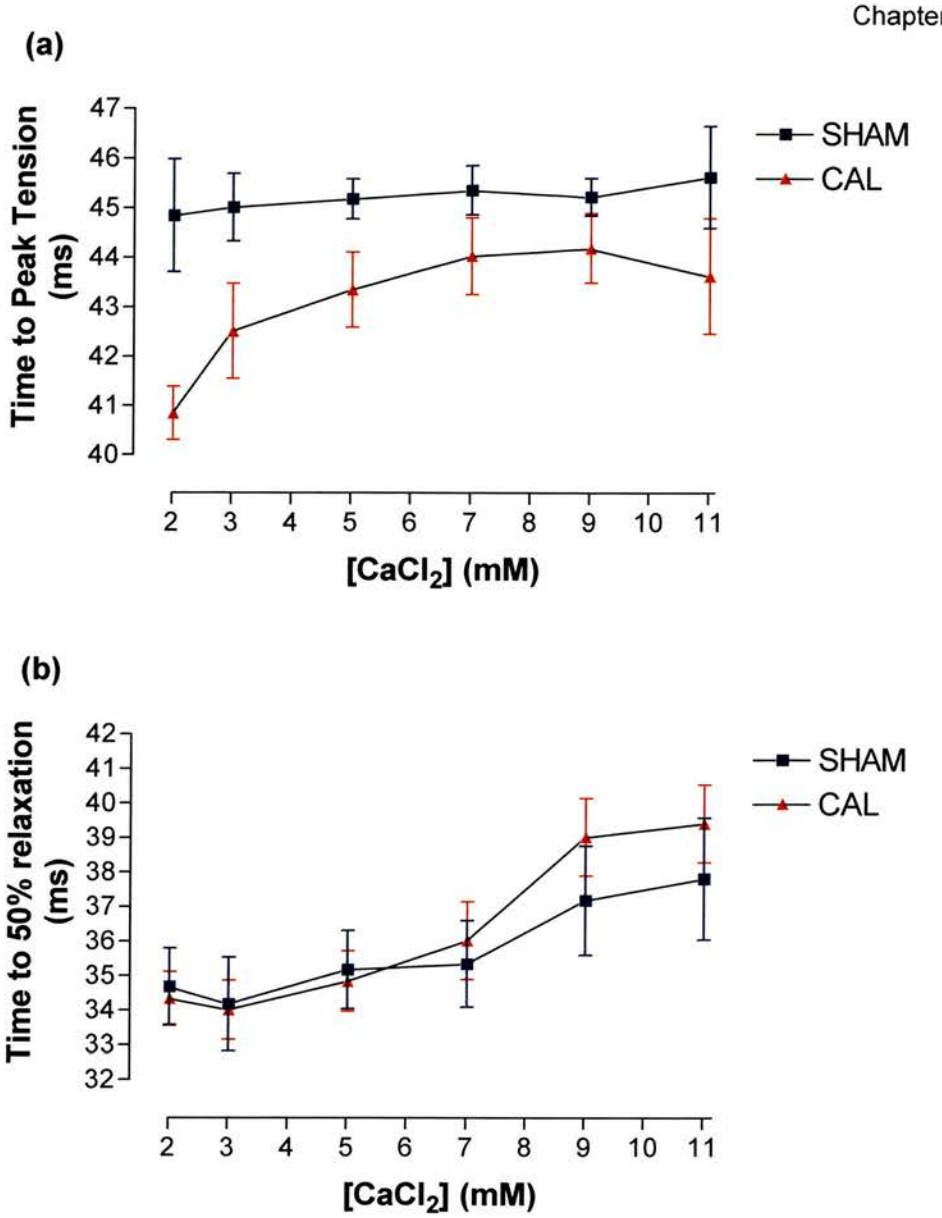


Figure 3.9. Effects of increasing extracellular Ca^{2+} concentrations on (a) TPT and (b) RT_{50} in papillary muscles from sham and CAL animals (n=6 in each group).

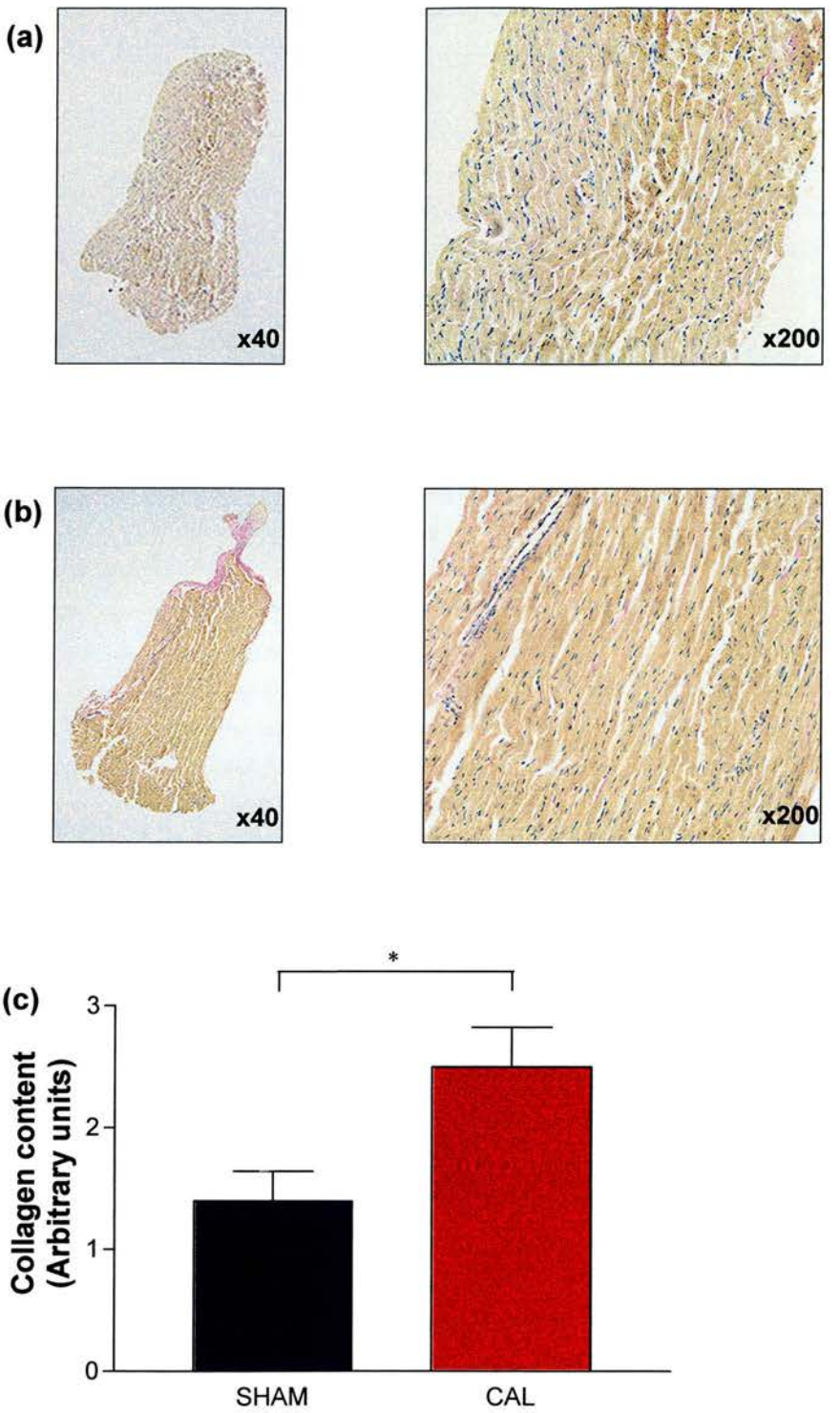


*Figure 3.10. Images of right ventricular papillary muscle from rats having undergone (a) sham-op surgery or (b) CAL for the induction of CHF. van Gieson's stain highlights collagen in pink and normal myocardium in yellow with nuclei counterstained dark blue with haematoxylin (x40 and x200 magnification), (c) histogram of collagen score (n=6 in each group, *P<0.05).*

3.3.8 Comparison of the ET system and ANP mRNA expression in failing and non-failing rat heart

Expression of ANP, the ET_A and ET_B receptors and ppET-1 mRNA levels were examined in the LV, septum and RV of sham-operated and CAL rats 15 weeks post MI (*Figure 3.11*). LV ANP/GAPDH mRNA expression was significantly elevated (1.4-fold) and correlated with collagen levels in the failing hearts ($r = 0.628$). A 1.6-fold increase of ANP mRNA was also detected in the septum of failing hearts, however, no change was measured in the RV. Densitometric analysis revealed that the ET_A mRNA was more abundant in myocardial tissues of sham animals compared to CAL, however, the differences were not statistically significant. In contrast, there was a tendency towards upregulation of ET_B receptor mRNA in the LV of the failing hearts but again, the differences were not statistically significant. No difference was observed in the level of ppET-1/ GAPDH mRNA expression in the LV, septum or RV from failing hearts.

Densitometric analysis of serial dilution data showed that GAPDH mRNA was not significantly different between sham and CAL rat hearts 15 weeks post MI. In contrast, ANP mRNA was elevated in each CAL tissue, confirming the semi quantitative observations.

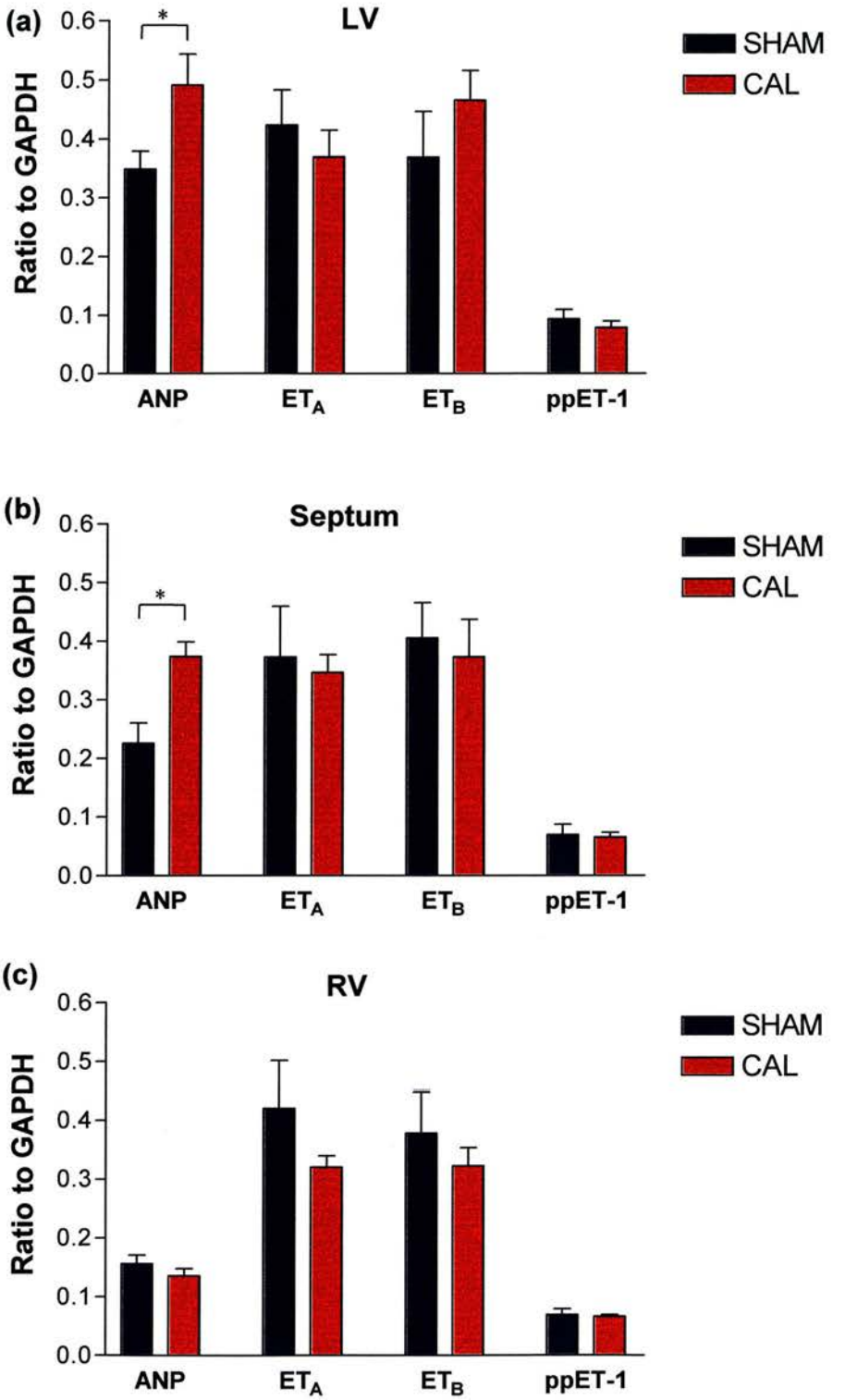


*Figure 3.11. Comparison of the relative intensities of ANP, ET_A, ET_B and ppET-1 / GAPDH mRNA levels from the (a) left ventricle, (b) septum and (c) right ventricle of failing and nonfailing rat hearts (n=6 in each group, *P<0.05).*

3.4 Discussion

The purpose of this study was to evaluate the role of cardiac ET receptors in the regulation of myocardial contractility in failing and nonfailing rat myocardium by examining the contractile behaviour of papillary muscles from sham and CAL rats and to establish whether a change in receptor density influenced the inotropic response in HF.

3.4.1 The effects of CAL

The deterioration of cardiac haemodynamic parameters observed in the chronically infarcted rat heart has previously been described (Pfeffer *et al.*, 1979). Characteristic elevation of LVEDP and reduced myocardial contractility (dP/dt_{max}) indicates that CAL rats had developed LVD. This is supported by the presence of cardiac hypertrophy, as evidenced by an increase in HW: BW, elevated interstitial collagen and elevated expression of LV ANP levels in CAL animals.

3.4.2 Passive and active length-tension relations

Mechanical stretch produced a more rapid rise of tension in passive papillary from CAL animals than sham-operated animals and the degree of stretch required to achieve L_{max} in the passive CAL papillary was less than that for sham-operated rats. These findings suggest a reduction of the myocardial elastic accommodation of CAL papillary muscles compared to the papillary from sham-operated. Correlation analysis confirmed that the length-tension relationship differed between the groups as the slope of the relationship was greater for the CAL papillary. The *y*-intercept was also greater for the CAL group, indicating an increased stiffness of the CAL myocardium compared to sham. Basal isometric contractile behaviour of CAL papillary was also altered. Peak developed tension was significantly reduced in the CAL group and was associated with an elevated resting tension, indicating myocardial dysfunction. Previous studies have observed a prolongation of TPT and RT_{50} in failing myocardium, most likely due to Ca^{2+} handling (Sandmann *et al.*, 1999). In contrast, baseline TPT and RT_{50} values in this study were not significantly

altered, however, the associated dP/dt_{max} and dP/dt_{min} of these animals was reduced, indicating LV dysfunction. Lowered contractility and elevated stiffness of the myocardium of infarcted animals can partly be attributed to an increased content of non-contractile material in the extracellular matrix, as evidenced here by an increased collagen content. Several studies have shown that the progressive development of cardiac fibrosis is accompanied by the accumulation of collagen type I and III (Weber *et al.*, 1988). This results in elevated stiffness of the noninfarcted hypertrophied myocardium and may be responsible in part for the deterioration of peak developed tension of the papillary muscle in the chronic state of MI induced heart failure. A possible relationship was demonstrated between the resting tension at L_{max} and the collagen content for the CAL group, where papillary muscles with high collagen content were associated with a high resting tension at L_{max}. However, the condition of linearity was not quite met. Increasing sample size may improve the statistical significance of the result.

3.4.3 Effects of β-adrenoceptor stimulation

Isoprenaline stimulation causes positive inotropic effects and a hastening of contraction and relaxation that are associated with cyclic AMP-dependent protein kinase phosphorylation of contractile proteins. Stimulation of the G-protein coupled β-adrenoceptor activates adenylyl cyclase which in turn activates PKA via cAMP to increase $[Ca^{2+}]_i$ during the plateau phase, this then enhances the intracellular calcium stores over the course of several beats. β-adrenoceptor activation also interacts with the pacemaker current channels to increase their open-state probability, thus increasing the depolarising rate and quickening contraction. In addition, the second messenger pathway increases the activity of phospholambam, the protein associated with the network sarcoplasmic reticulum (SR) that is responsible for regulating the activity of the SR Ca^{2+}-ATPase (SERCA) pump, to accelerate the re-uptake of Ca^{2+} by the SR and shorten the period of relaxation. A tendency for a reduction in the positive inotropic response to isoprenaline in the CAL group is consistent with the previously described down regulation and desensitisation of β-adrenoceptors in heart failure (Bristow *et al.*, 1993).

3.4.4 The effects of exogenous ET-1

Previous studies using multicellular preparations have described the positive inotropic actions of ET-1 occurring in a simple concentration-dependent manner in animal (Li *et al.*, 1991; Kasai *et al.*, 1994) and human myocardial preparations (Meyer *et al.*, 1996; Pieske *et al.*, 1999). In contrast, consistent responses to direct ET-1 application were not achieved in this study, instead, a biphasic tendency was observed in the sham group. At low concentrations of exogenous ET-1 a decrease of tension was observed. This was possibly due to relaxation induced by endothelial cell ET_B receptor stimulated release of nitric oxide (NO). At higher ET-1 concentrations a positive inotropic response was predominant, mediated by ET_A and possibly ET_B receptors expressed on cardiomyocytes. However, in the present study, responses from sham papillary muscles were variable and although the results appeared biphasic, the changes were not statistically significant.

In a recent study using a similar protocol Qi *et al.* (2001) reported similar biphasic responses to ET-1 in normal rat papillary muscles and attributed them to receptor distribution. However, in contrast to the current study, Qi *et al.* (2001) observed less variation of responses in the control group. There are several potential explanations for the differences between the Qi *et al.* (2001) study and the current study. Bathing $[Ca^{2+}]$ and the bath temperature were both higher in the current study (2mM and 37°C compared to 0.7mM and 29°C respectively) and sample size was greater in the Qi *et al.* (2001) study. In addition, a previous study established that removal of the endothelial layer by brief immersion in the detergent Triton did not alter the level of tension induced by ET-1 but shifted the dose-response curve to the left, decreasing the threshold at which ET-1 has myocardial effects (Li *et al.*, 1991). These results indicate that endocardial endothelial ET_B receptors may modify the ET-1 induced twitch configuration characteristics via stimulation and release of NO. Variability in endocardial endothelial integrity could therefore account for some of the variability observed in papillary muscles from sham-operated animals the present study.

In the current study, exogenous ET-1 had no significant influence on the contractile state of papillary muscles from CAL animals. Although a slight biphasic response

was observed, responses were not significantly altered by increasing ET-1 concentration and were not as exaggerated as the biphasic response of sham-operated papillary muscles. The lack of consistent relaxation at lower concentrations of exogenous ET-1 again indicated the loss of an endothelial-mediated mechanism in CAL papillary muscles. Potential loss of the ET_B mediated release of NO may have resulted from variable endocardial endothelial integrity due to mechanical transfer, or could have resulted from the endothelial dysfunction that occurs in heart failure. Similarly, Qi *et al.* (2001) reported that low concentrations of exogenous ET-1 had no effect on papillary muscles from infarcted hearts. However, in contrast to the current study, responses to higher ET-1 concentrations in the CAL group were significantly increased compared to the sham-operated group of papillary muscles (Qi *et al.*, 2001). The differences between the current study and the Qi *et al.* (2001) study are most likely attributed to the larger sample size used and the condition that was employed, where only papillary from hearts with infarcts of a specified size range (30-45%) were used (Qi *et al.*, 2001).

3.4.5 Stretch and ET-1

ET-1 is synthesised in endocardial endothelial cells and in cardiomyocytes. Another possible explanation for the inconsistent results in the present study could be that endogenous ET-1 was released upon stretch (Kuchan & Frangos, 1993; Macarthur *et al.*, 1994; van Wamel *et al.*, 2001). Release of ET-1 through an autocrine-paracrine mechanism would have increased the local concentration and led to receptor occupation at the level of the myocyte. Once bound, the peptide was capable of causing stimulation for over 40 minutes and masking the effects of additional binding by exogenous ET-1. A possible relationship between the resting tension at L_{max} and the peak response to exogenous ET-1 was demonstrated by linear regression analysis. CAL responses produced a negative correlation slope, where high resting tension was associated with a reduced response to exogenous ET-1, however, the condition of linearity was not met. The degree of stretch required to achieve peak isometric tension was greater for CAL papillary muscles compared to sham-operated muscles and resulted in an increased resting tension. Therefore, the degree of mechanical stretch may have influenced the amount of endogenous ET-1 released

and this would have influenced the availability of free receptors not bound by endogenous ET-1. However, no significant difference in response to exogenous ET-1 was observed between sham-operated and CAL papillary muscles. Using a larger sample size and imposing a condition, which would exclude rats with infarcts under a specified size, as many studies do, would provide a more accurate correlation. However, papillary muscles would still have to be stretched to normalise forces, therefore, the use of mixed or selective antagonists would be required to exclude the influence of endogenous ET-1.

3.4.6 The intracellular mechanisms of ET-1 action

ET-1 produced positive inotropic effects at higher concentrations in most papillary muscles but was less consistent at producing a response at lower concentrations. The positive effects were induced by a rise in $[Ca^{2+}]_i$ via complex intracellular pathways. The mechanisms underlying ET-1 receptor-induced $[Ca^{2+}]_i$ rise are not completely understood, however, patch-clamp studies have shown ET-1 to be involved in activating voltage operated and voltage independent Ca^{2+} channels in cultured cells (Inoue *et al.*, 1990; Miwa *et al.*, 1999). In a recent study, Hong (2002) demonstrated that ET-1 stimulates two phases of $[Ca^{2+}]_i$ increase in cultured myocardial ventricular cells, the first results from the entry of external Ca^{2+} via L-type channels while the secondary phase induced by ET-1 is a result of both external Ca^{2+} influx and internal Ca^{2+} release from Ca^{2+} stores.

ET-1 is capable of influencing other ionic currents. In a previous study the effectiveness of ET-1 to stimulate Na^+/H^+ exchanger activity was impaired in rat cardiac hypertrophy, resulting in reduced functional effects of ET-1 (Ito *et al.*, 1997). This was partly due to the failure to develop intracellular alkalinisation, required to sensitise the contractile proteins. Furthermore, as previously described, the L-type Ca^{2+} current (I_{CaL}) is the main depolarising current contributing to the plateau-shape of the cardiac action potential and governs release of Ca^{2+} from the sarcoplasmic reticulum (SR) (Cleemann & Morad, 1991). I_{CaL} has been found to be stimulated by ET-1 (Lauer *et al.*, 1992; Bkaily *et al.*, 1995) and is downregulated in heart failure

(Boixel *et al.*, 2001). Conversely some studies have shown that the ET_A receptor subtype is coupled to a decrease in I_{Ca} via L-type Ca^{2+} channel inhibition, possibly to protect against ventricular arrhythmias and myocardial Ca^{2+} overload (Ono *et al.*, 1994; Kelso *et al.*, 1998). Therefore, this suggests that alterations of the subcellular mechanisms that mediate the functional responses of ET-1, may contribute to reduce the inotropic effect in diseased myocardium and counterbalance any potentiated effect induced by an increased ET_A or ET_B receptor density. In the present study, the presence of LVD did not modify the inotropic response to ET-1. However, Qi *et al.* (2001) and others have reported potentiated contractile responses to ET-1 in isolated tissues from failing hearts. The difference in the two studies could be attributed to the severity of experimental heart failure or the time course of the experiment. In the Qi *et al.* (2001) study, animals exhibited moderate to severe HF as indicated by a more elevated LVEDP, reduced dP/dt_{max} and moderate to large infarct sizes, all parameters more pronounced than the present study. In addition, the animals were younger and were used at 4 weeks post MI compared to 15 weeks in this study. Furthermore, it is likely that receptor distribution and other factors change with time and over the course of CHF development.

Consistent with other studies, ET-1 had no effect on twitch configuration characteristics, unlike isoprenaline, consistent with the influence of ET being mediated via a different mechanism. β-adrenoceptor stimulation activates the pacemaker current (I_{Na}) via cAMP to increase the depolarisation rate and decrease the TPT, however, there is no evidence to suggest that ET-1 modulates the I_{Na} (Bkaily *et al.*, 1995). In addition, isoprenaline stimulation shortens RT_{50} by increasing re-uptake of Ca^{2+} into SR stores by activating the SERCA pump via phospholambam whereas ET-1 does not.

3.4.7 The influence of exogenous Ca^{2+} concentration

In normal myocardium, an increase of extracellular Ca^{2+} produced typical concentration-dependent responses in isometric twitch tension. Ca^{2+} enters the myocyte through voltage-gated dihydropyridine-sensitive sarcolemmal Ca^{2+} channels during depolarisation, this local pool is sufficient to activate adjacent ryanodine

receptors to release Ca^{2+} from the SR. This punctate increase of Ca^{2+} is termed Ca^{2+} spark, according to its visualisation by confocal microscopy (Cheng *et al.*, 1993) and cytosolic increase of $[Ca^{2+}]$ causes activation of the contractile proteins. During relaxation Ca^{2+} is then removed from the cytosol into the SR by the SERCA pump and across the sarcolemma, mainly by the Na^{+}/Ca^{2+} exchanger (Barry & Bridge, 1993). A gradual increase of SR loading occurs, resulting in an elevated quantity available for the next contraction. The maximum amplitude of isometric force in sham papillary was obtained at 9mM Ca^{2+}. In contrast, isometric twitch tension was reduced in the failing papillary and required 11mM extracellular Ca^{2+} to reach a maximum. Furthermore, the present study showed that TPT and RT_{50} values tended to increase after addition of extracellular Ca^{2+}. The change in sensitivity to Ca^{2+} in the CHF group indicates an alteration in intracellular Ca^{2+} handling which is associated with disturbed contractile function. Indeed, it has been shown that the pharmacology of the I_{Ca} is altered in heart failure and hypertrophy. The density of L-type Ca^{2+} channels has been shown to decrease in moderate to severe stages of CHF in rats, as evaluated by antagonist binding studies (Dixon *et al.*, 1990; Gopalakrishnan *et al.*, 1991). Furthermore, patch-clamp studies of isolated rat CHF myocytes have demonstrated that I_{CaL} showed both a significantly reduced peak amplitude and a slower inactivation compared with myocytes from sham-operated animals (Aimond *et al.*, 1999; Boixel *et al.*, 2001). Additionally, mRNA and protein levels of the SERCA pump, which is crucial for Ca^{2+} accumulation within the SR, were found to decrease continuously with increasing severity in the rat model of CHF (Zarain-Herzberg *et al.*, 1996). Therefore, the results from the current study are consistent with the reports that failing myocardium is associated with altered Ca^{2+} handling via changes in the density and function of several Ca^{2+} regulatory proteins (for review see Hasenfuss, 1998b).

3.4.8 Expression of ET-system and ANP mRNA

Previous studies in our laboratory using *in situ* hybridisation have demonstrated a localised increase of the ET_B receptor subtype in the LV surrounding the infarcted region (Sherry, 2000; Smith *et al.*, 2000). In the current study, ET_B receptor mRNA expression was greater but not significantly increased in the failing LV tissue,

suggesting that ET_B receptor expression may exhibit a marked increase in the tissue nearest the scar but this is not necessarily reflected throughout the LV myocardium. Another possibility is that ET_B mRNA is increased in cardiomyocytes while ET_B receptor mRNA in endocardial endothelial cells has decreased, therefore counterbalancing the result. No significant change in the expression pattern for the ET_A receptor was observed in CAL animals, contrasting with previous studies (Sakai *et al.*, 1996b; Tønnessen *et al.*, 1997; Picard *et al.*, 1998). Plasma ET-1 and big ET-1 levels were not measured in this study, as previous investigations in our laboratory have consistently shown no change in concentration in CAL animals (Sherry, 2000). In the current study, ppET-1 mRNA expression remained unchanged, in agreement with our previous findings but again in contrast with the other published data (Sakai *et al.*, 1996b; Tønnessen *et al.*, 1997; Picard *et al.*, 1998). The discrepancy between this study and the other reports is most likely due to the severity of LVD resulting from CAL.

In conclusion, it has been demonstrated that the papillary muscle technique is a suitable preparation for the evaluation of contractile function in heart failure. However, investigation of the contribution of the ET-system to cardiac contraction following CAL was restricted most likely due to the influence of stretch-released endogenous ET-1. Furthermore, no clear pattern emerged to define the role of the ET_B receptor in the regulation of cardiac contraction in the infarcted heart. Additionally, results indicated that the severity of experimental heart failure influenced the contractile responses to ET-1, suggesting that a more accurate assessment would require imposing a condition of inclusion on the basis of infarct size. Further investigation is required to define the contractile role of ET receptors in heart failure.

Chapter 4

Investigation of cardiac ET receptor subtype(s) responsible for contraction in normal myocardium

4.1 Introduction

In the normal rat heart, both ET_A and ET_B receptors are diffusely distributed throughout the atrial and ventricular myocardium. In our laboratory, previous *in situ* studies have revealed an 80:20 distribution of ET_A:ET_B receptors in the normal left ventricle (LV) (Sherry, 2000), while other studies have reported 85:15 (Touyz *et al.*, 1996) or a 90:10 distribution of ET_A:ET_B receptors (Fareh *et al.*, 1996; Sakai *et al.*, 1996a) in normal rat LV. The positive inotropic effects of ET-1 have consistently been observed in atrial and ventricular tissue preparations from many species, including humans (Davenport *et al.*, 1989; Moravec *et al.*, 1989; Meyer *et al.*, 1996). However, there is a distinct lack of clarity regarding the contribution of the ET_B receptor subtype in the regulation of cardiac contractility. Some antagonist studies have suggested that the predominant ET_A receptor subtype is solely responsible for mediating the positive responses of ET-1 in the heart (Meyer *et al.*, 1996; Pönicke *et al.*, 1998), while others have demonstrated that both receptor subtypes are involved (Kasai *et al.*, 1994; Beyer *et al.*, 1996).

In the previous chapter (Chapter 3), *in vitro* contractile responses to direct application of agonist failed to provide consistent results. The influence of ET receptor subtypes on contraction was complex and endogenous ET-1, potentially released by stretch, may have confused the responses in papillary muscles from sham-operated as well as CAL animals. Therefore, a study was designed to assess contractility in papillary muscles from control animals using an antagonist approach, to identify the contribution of endogenous ET-1 to normal contraction.

The aims of this chapter were to develop a protocol that may be used to better define the role of receptors in heart failure. The role of ET_A and ET_B receptors in the regulation of normal contractility were investigated by determining the influence of endogenous ET-1, using the ET_A receptor selective antagonist, BQ123, the ET_B receptor selective antagonist, BQ788, or a combination of both.

4.2 Methods

Male Wistar rats (390-590g, Charles River) were weighed and anaesthetised with sodium pentobarbital (60mgkg^{-1}, i.p. Sagatal). The hearts were rapidly excised and placed in Tyrode's solution to expel blood from the ventricles prior to immersion in ice-cold cardioplegic solution. After removing the atria and large vessels, the heart was weighed. The right ventricle (RV) was dissected open and the papillary muscles were carefully dissected out and vertically mounted in carbogen bubbled 2,3-butanedione-monoxime (BDM)-Tyrode's solution (pH 7.4, 37°C) as described before in section 2.2.2. Papillary muscles were incubated with one of four different bath conditions; 1, vehicle, bovine serum albumin (BSA 0.1%); 2, BQ123 (10^{-6} M); 3, BQ788 (10^{-6} M); 4, BQ123/BQ788, randomised by a Latin square method.

After an initial incubation period of 20 minutes, the passive length/tension relationship was investigated. The preparation was then returned to slack and the bath solution replaced with Tyrode's solution. Treatments were re-administered and the preparation was stimulated (3Hz, 20% above threshold voltage). The papillary was left to equilibrate (20 minutes) before the active length/tension relationship was investigated to L_{max}. Two separate functional studies were carried out using the antagonist conditions described above.

4.2.1 Functional study 1 - January/February

After a period of stabilisation, a cumulative concentration-response curve for isoprenaline (10^{-9}-10^{-6}M) was performed. Following washout, replacement of treatment and stabilisation, the muscle was challenged with a single 10^{-8}M dose of ET-1. Finally, after a subsequent washout, $CaCl_2$ was added cumulatively ($2x10^{-3}$-10^{-2} M). All forces were normalised by cross sectional area (CSA) of the papillary muscle. Subsequently, due to encouraging observations in study 1, a second study was designed to (i) increase sample size and (ii) look at the interaction of ET-1 and Ca^{2+} in more detail.

4.2.2 Functional study 2 - July/August

The protocol for this study was the same as described above for study 1, with an additional Ca^{2+} concentration-response curve ($2x10^{-3}$ - 10^{-2}M) after isoprenaline stimulation and before the application of ET-1. All forces were normalised by CSA of the papillary muscle.

4.2.3 Molecular study

Tissue samples (approximately 100mg) of the RV free wall, LV free wall and the septum were taken from both functional studies, snap frozen in liquid nitrogen and stored at -70°C for subsequent analysis of gene expression. Semi-quantitative RT-PCR was performed as described in section 2.3 using 2μg of mRNA to investigate the atrial natriuretic peptide (ANP), ET_A, ET_B and ppET-1/GAPDH mRNA ratios. All samples were processed under the same conditions concurrently and PCR products of a gene were evaluated densitometrically on the same agarose gel.

Comparison between groups was performed by a one-way or two-way analysis of variance where appropriate, followed by Bonferroni's multiple comparisons test. A P value <0.05 was considered significant.

4.3 Results

4.3.1 Basal responses

Papillary muscles stretched under passive conditions produced typical length/tension relationship curves. No significant difference was observed between treatment groups of each study (results not shown). No significant difference in CSA was reported between groups. January/February study: vehicle, 0.33 ± 0.04mm^2; BQ123, 0.44 ± 0.07mm^2; BQ788, 0.49 ± 0.11mm^2 and mixed, 0.50 ± 0.10, n=8, P=0.43; July/August study: vehicle, 0.34 ± 0.05mm^2; BQ123, 0.33 ± 0.03mm^2; BQ788, 0.30 ± 0.03mm^2 and mixed, 0.35 ± 0.05mm^2, n=9, P=0.82.

As expected, progressive stretch of electrically stimulated papillary muscles produced characteristic increases in developed tension. The peak isometric tension achieved at L_{max} was significantly different between the vehicle and mixed antagonist groups from study 1, while no significant difference was observed between groups from study 2 (*Figure 4.1*). No significant difference in resting tension was reported between groups of either study. Antagonist treatment had no significant effect on baseline time to peak tension (TPT) or time from peak to 50% relaxation (RT_{50}) values in either study (*Table 4.1*).

4.3.2 Influence of isoprenaline in the presence of endothelin antagonists

The positive inotropic effects of β-adrenergic stimulation were demonstrated in papillary muscles of all treatment types and were associated with the hastening of timing parameters (TPT and RT_{50}) (results no shown). In study 1, the antagonist treatments had no significant influence on the response to isoprenaline, although there was a tendency toward reduced responsiveness in the presence of the mixed antagonist compared to vehicle (*Figure 4.2a*). In contrast, in study 2 no difference was observed against the vehicle group but there was a significant difference between antagonist groups (*Figure 4.2b*). Responses to isoprenaline in the presence of the ET_B selective antagonist, BQ788, were significantly reduced at higher concentrations (10^{-7}-10^{-6}M) compared to the ET_A selective antagonist and mixed groups. Antagonist treatment had no significant effect on the shortening of contractile parameters in response to isoprenaline stimulation (results not shown).

4.3.3 Contractile responses to exogenous ET-1 in papillary muscles pre-incubated with ET antagonists

Papillary muscles in the vehicle group of study 1 produced positive inotropic responses to exogenous ET-1. Although papillary muscles treated with $ET_{A/B}$ antagonist tended towards reduced responses, there was no significant difference between these groups due to the wide variation of responses (*Figure 4.3a*). The effects of ET-1 in study 2 were again varied and no difference was found between

groups (*Figure 4.3b*). No change was observed in contractile timing parameters (TPT or RT_{50}), consistent with previous studies of normal myocardium.

Table 4.1. TPT and RT_{50} values of electrically stimulated right ventricular papillary muscles preincubated with ET antagonists in Study 1 and Study 2.

	Study 1 (n=8)		Study 2 (n=9)	
	TPT (ms)	RT_{50} (ms)	TPT (ms)	RT_{50} (ms)
Vehicle	57.7 ± 1.3	44.2 ± 2.6	50.2 ± 1.6	40.1 ± 2.4
BQ123	53.0 ± 1.7	40.2 ± 1.9	52.2 ± 1.9	41.8 ± 1.7
BQ788	54.8 ± 3.2	46.8 ± 2.5	51.5 ± 1.3	40.2 ± 1.6
Mixed	50.3 ± 2.7	41.5 ± 0.9	51.9 ± 1.2	40.7 ± 1.2

Values are means ± SEM. Study 1, January/February; Study 2, July/August; TPT, time to peak tension; RT_{50}, time from peak to 50% relaxation.

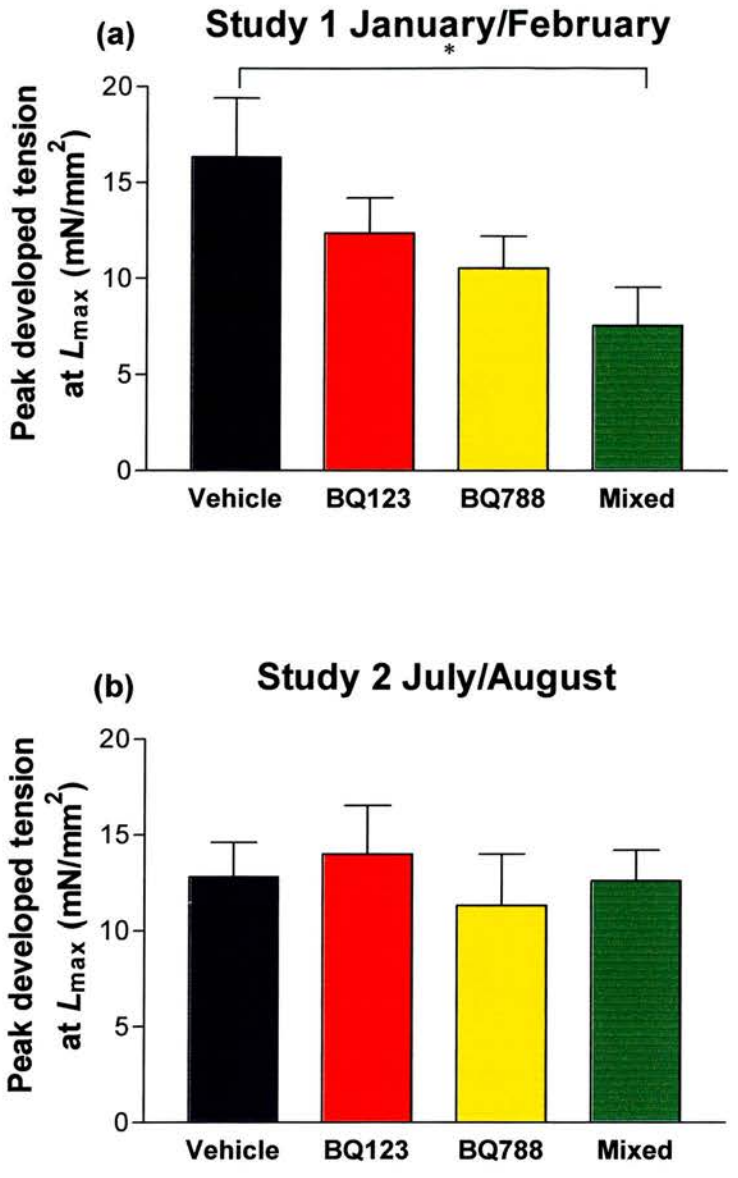


*Figure 4.1. Comparison of peak developed tension at L_{max} achieved by papillary muscles from normal animals after pre-incubation with ET antagonists (a) Study 1, January/February (n=8, *P<0.05), (b) Study 2, July/August (n=9).*

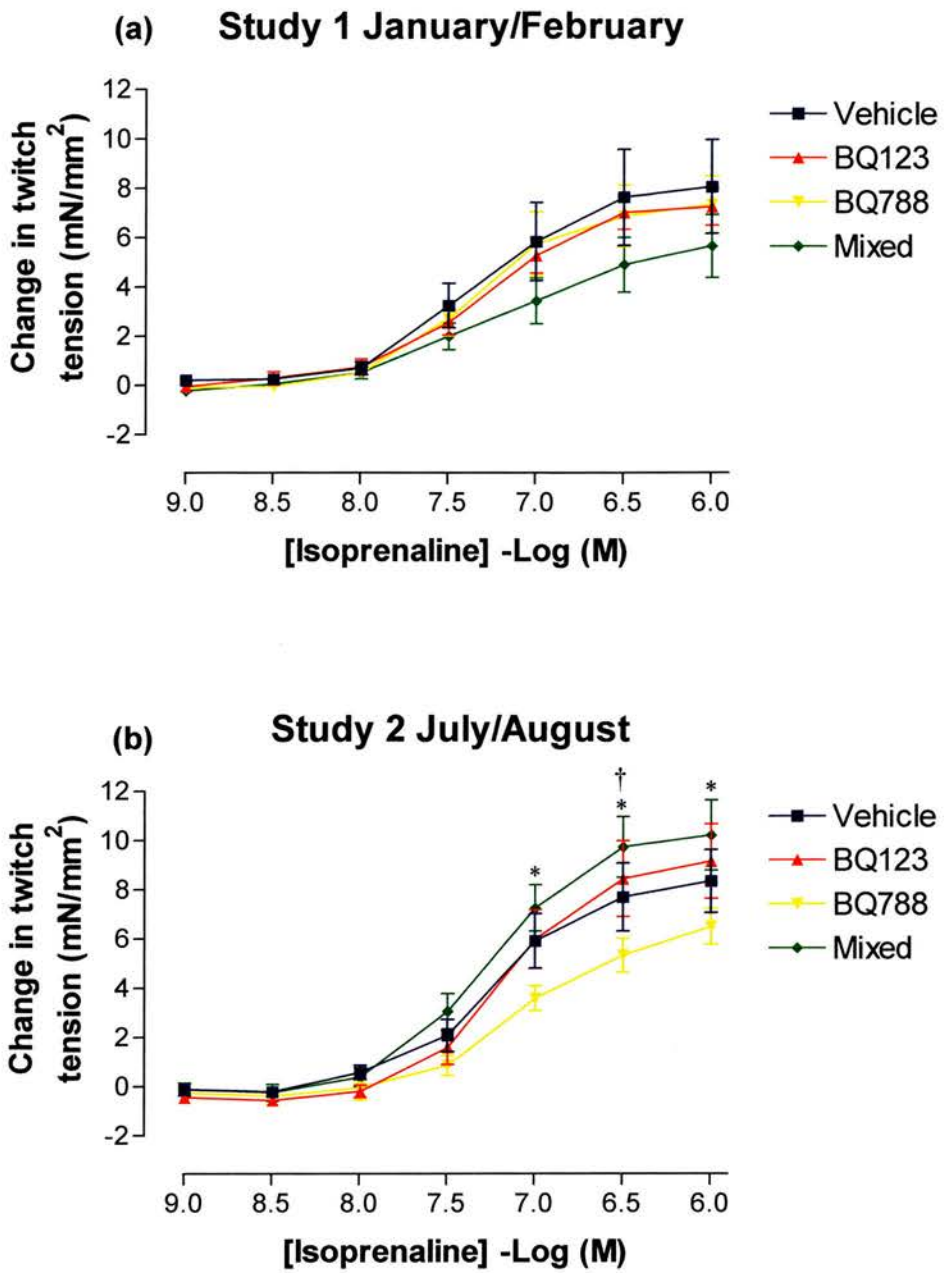


*Figure 4.2. Positive inotropic responses to isoprenaline in papillary muscles pre-incubated with ET antagonists (a) Study 1 (n=8), (b) Study 2 (n=9, *P<0.01 BQ788 compared to mixed, †P<0.05 BQ788 compared to BQ123).*

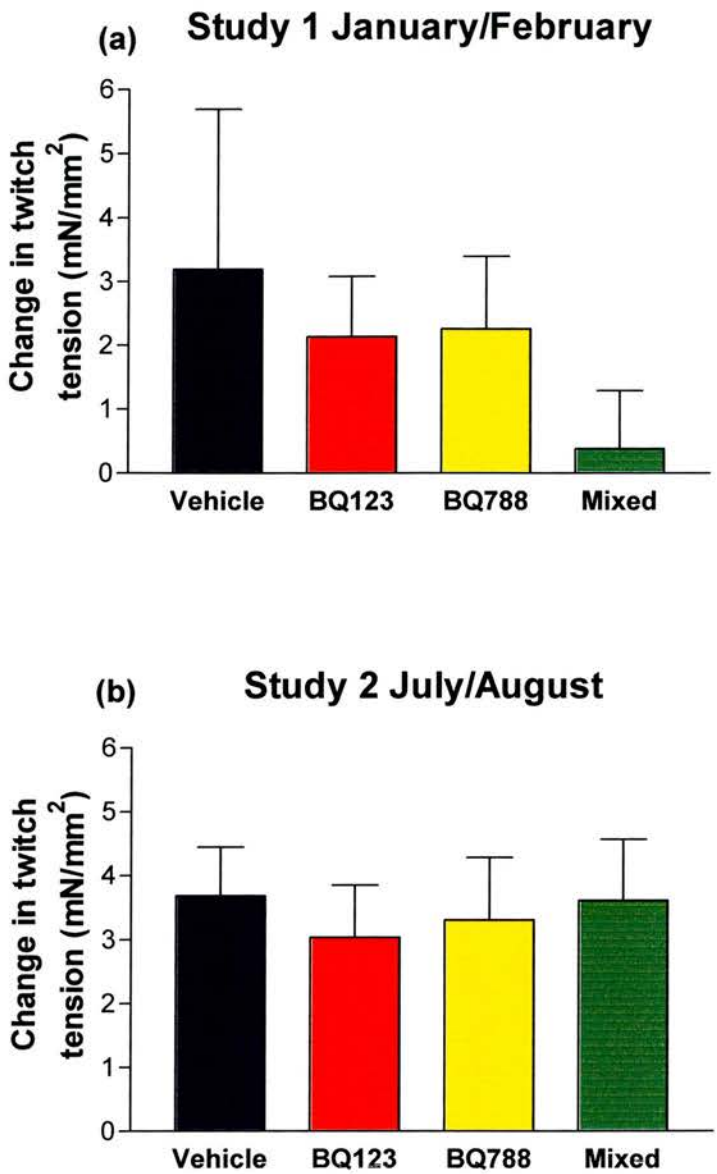


Figure 4.3. Change in twitch tension of papillary muscles in response to exogenous ET-1 (10^{-8}M) in the presence of ET antagonists (a) Study 1 (n=8), (b) Study 2 (n=9).

4.3.4 Effects of extracellular Ca^{2+} on contractile behaviour

An increase of extracellular Ca^{2+} concentration produced positive inotropic effects and no alteration of contractile timing parameters. Results from study 1 showed that responses to increasing bathing [Ca^{2+}] were significantly reduced in the antagonist groups compared to the vehicle group. In addition, peak response to extracellular Ca^{2+} was achieved at 6mM for the vehicle group and 7mM for the antagonist groups (*Figure 4.4a*). Responses in study 2 were not modified by the presence of ET antagonists (*Figure 4.4b*), while maximum developed tension was achieved in all groups with 7mM Ca^{2+}. No difference in TPT or RT_{50} was observed from either study (results not shown). In study 2, no difference in contractile response to Ca^{2+} was observed when evaluated before or after the administration of ET-1 (results not shown).

4.3.5 Seasonal comparison of the ET system and ANP mRNA expression in cardiac tissues from control animals

Due to the variation of contractile responses between the two studies, GAPDH, ANP, the ET_A and ET_B receptors and ppET-1 mRNA expression levels were examined in the LV, septum and RV of hearts from study 1 and 2 (*Figure 4.5*). The two sets of functional experiments were conducted 6 months apart, study 1 in January/February and study 2 in July/August. ANP/GAPDH mRNA expression was significantly elevated in septum and RV tissue samples from study 2 compared to study 1 ($P<0.05$). The ratio of ANP/GAPDH mRNA also tended to be elevated in the LV sample of study 2, however, the result was not significant. The expression ratio of ET_A/GAPDH mRNA was significantly greater in all myocardial samples from study 2, while, ET_B/GAPDH mRNA was also increased, showing statistical difference in the septum and the RV myocardial tissues. In addition ppET-1 was significantly elevated in the septum and RV of study 2 compared with study 1. No change in GAPDH mRNA expression was observed between groups as evidenced by densitometric intensities of GAPDH bands.

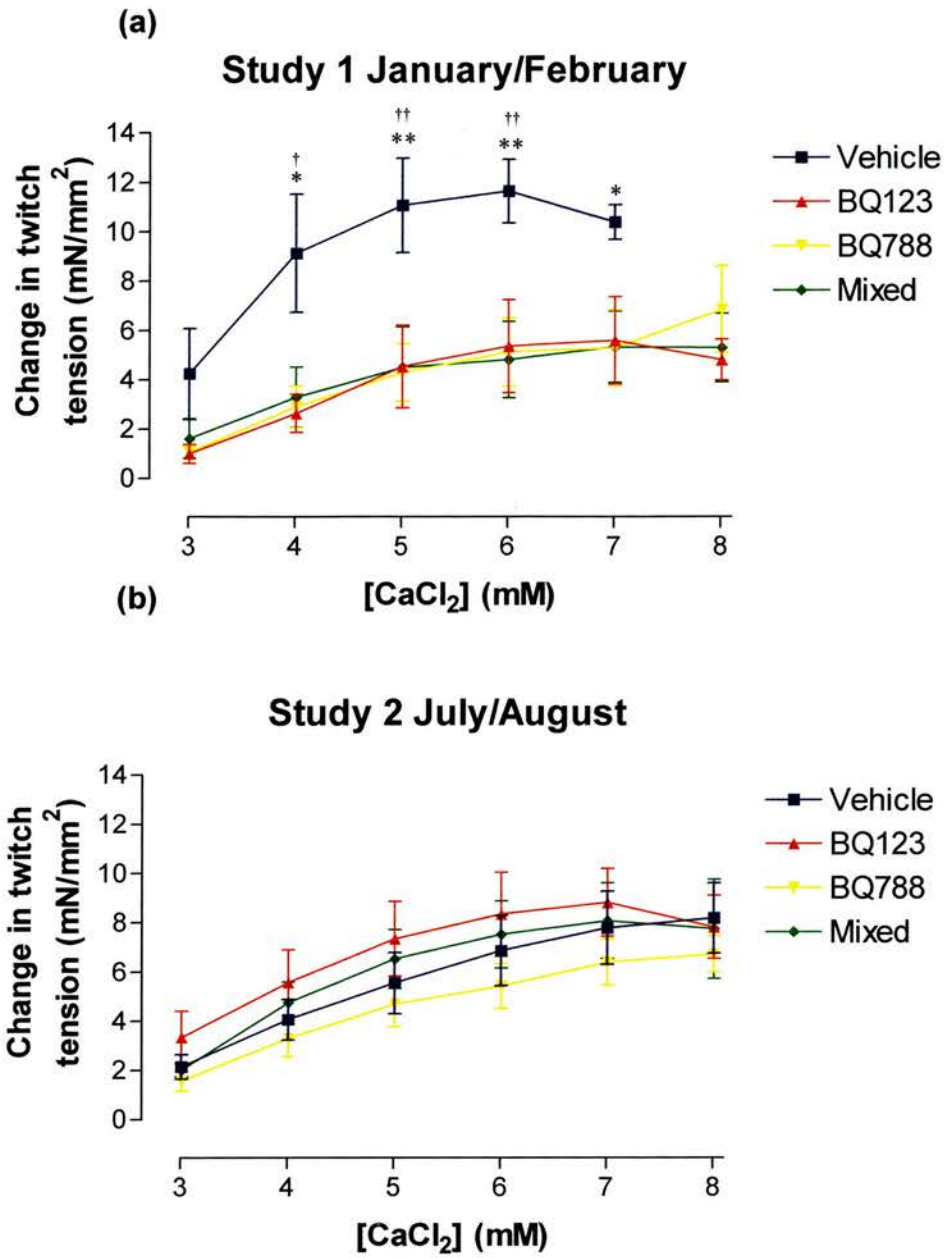


*Figure 4.4. The influence of extracellular Ca^{2+} on twitch tension of papillary muscles pre-incubated with ET antagonists in (a) Study 1 (n=5, *P<0.01, **P<0.001, vehicle versus BQ123; †P<0.05, ††P<0.01, vehicle versus BQ788 and mixed) and (b) Study 2 (n=8).*

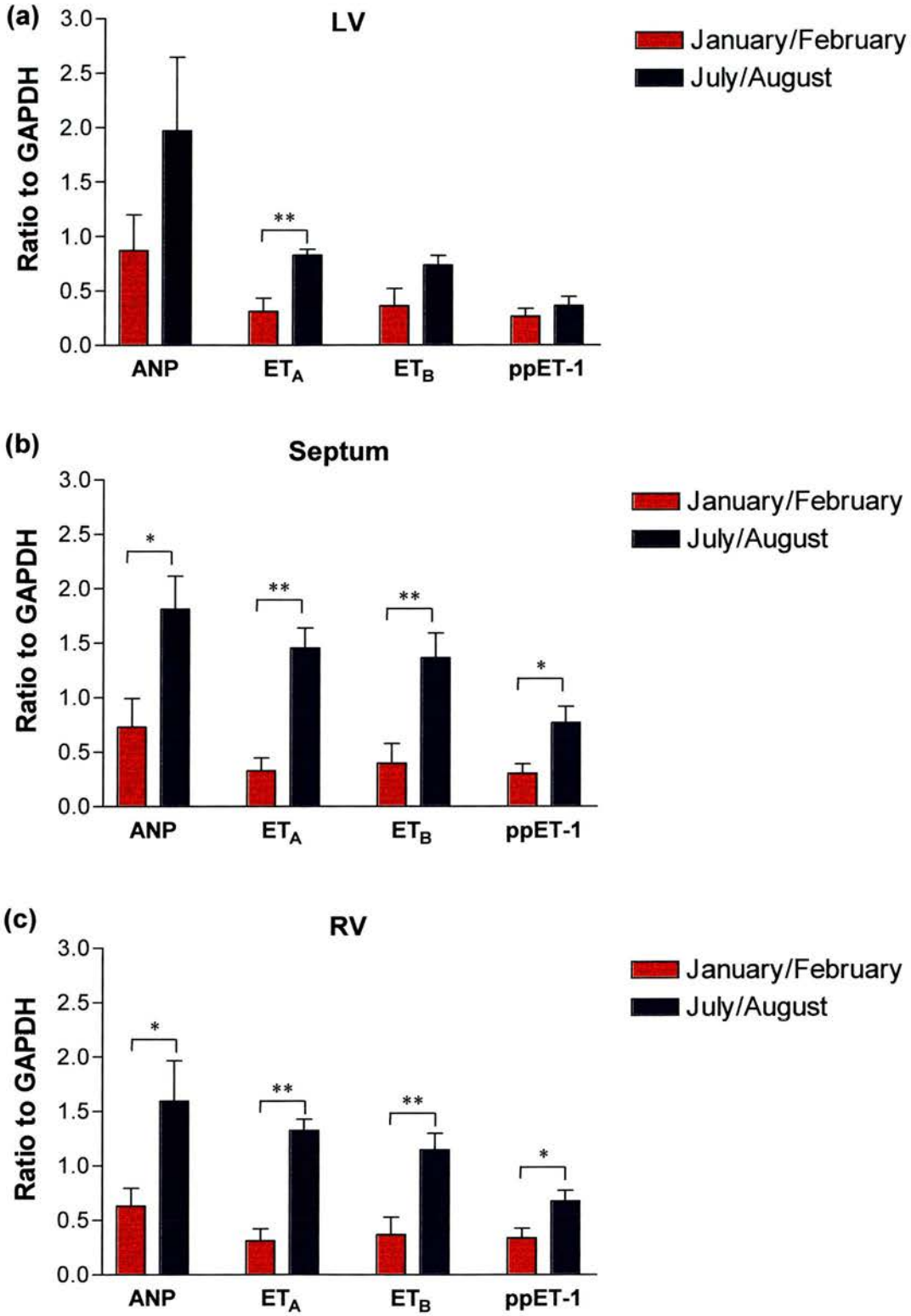


*Figure 4.5. Comparison of the relative intensities of ANP, ET_A, ET_B and ppET-1 mRNA/ GAPDH mRNA levels from the (a) left ventricle (LV), (b) septum and (c) right ventricle (RV) of normal rat hearts used in Study 1 (Jan/Feb) and Study 2 (July/Aug) (n=5, *P<0.05, **P<0.01).*

4.4 Discussion

The purpose of this investigation was to develop an effective protocol to evaluate the functional influence of ET receptors on cardiac contractility in normal rat myocardium and to determine the role of endogenous ET-1 using selective ET receptor antagonists. The expression of the ET system was also investigated.

As discussed in the previous chapter (Chapter 3, section 3.4.5), release of endogenous ET-1 from endothelial cells and cardiomyocytes can be induced by mechanical stretch (Kuchan & Frangos, 1993; Macarthur *et al.*, 1994; van Wamel *et al.*, 2001) and may have contributed to the inconsistent results observed when using agonist. Therefore, ET antagonists were included in the current protocol to block the influence of stretch released endogenous ET. Myocardial ET receptors are coupled via G-proteins to PLC-IP_3 and -DAG intracellular cascades that induce ET-1 stimulated release of Ca^{2+} from intracellular stores, increase the inward Ca^{2+} current (I_{Ca}) through L-type channels and increase myofilament sensitivity. Therefore, blockade of ET receptors should result in a reduction of the background inotropic influence provided by endogenous ET-1 via reduced intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$).

Baseline results from study 1 suggest that ET receptors are involved in regulating basal contractile force as a significant reduction in peak active tension was observed in the mixed antagonist group compared to the vehicle group. This observation also suggests that endogenous ET-1 was released within the myocardium, perhaps by stretch, consistent with other studies (Alvarez *et al.*, 1999; van Wamel *et al.*, 2001). However, selective receptor blockade was not sufficient to produce a significant reduction in isometric tension. ET_A blockade was expected to reduce the response more than ET_B blockade due to the predominance of ET_A receptors (80%) (Sherry, 2000) in rat myocardium. The fact that neither selective antagonist reduced contraction alone suggests the possibility of receptor crosstalk, where one receptor compensates when the other receptor is blocked, as previously demonstrated in vascular tissue (Mickley *et al.*, 1997).

The positive inotropic responses to isoprenaline in study 1 were not significantly altered by the bath presence of ET antagonists. However, a slight reduction of responses in the mixed group suggests an interaction between β-adrenergic receptors and ET receptors in the normal rat heart.

In study 1, the positive inotropic response to exogenous ET-1 was not significantly reduced by the presence of ET antagonists. Incubation with selective ET receptor antagonists was not effective, only the mixed antagonist group tended towards a reduced inotropic response compared to the vehicle group. However, large variations in responses to exogenous ET-1 were observed, possibly due to receptor occupation by endogenous ET-1 or to failure of the antagonists to exert their actions.

Responses to increasing concentration of extracellular Ca^{2+} in study 1 suggest that both receptors, activated by endogenous ET-1, are involved in mediating positive inotropism. Papillary muscles in the vehicle group produced a greater change in twitch tension, compared to those achieved by the antagonist groups. This is consistent with reports that ET receptors alter the force of contraction by influencing Ca^{2+} handling (Touyz *et al.*, 1996; Kelso *et al.*, 1998; Pieske *et al.*, 1999; Boixel *et al.*, 2001; Hong, 2002). However, an alternative explanation could be that the antagonist had a direct negative inotropic effect, which could explain the reduction in the response to Ca^{2+} without involving endogenous release of endothelin.

As discussed before, ET-1 does not influence the pacemaker current or the rate of Ca^{2+} re-uptake by intracellular stores (Chapter 3, section 3.4.6), therefore as expected, bath treatments had no effect on baseline contractile timing parameters TPT or RT_{50}. Isoprenaline induced a concentration-dependent decrease in TPT and RT_{50}, consistent with β-adrenoceptor stimulation that were not altered by the presence of ET antagonists. Similarly, ET antagonists had no effect on TPT and RT_{50} values in response to exogenous ET-1 or extracellular Ca^{2+}.

Therefore, in summary, contractile behaviour of papillary muscles from control animals in study 1 suggested that both ET receptors are involved in regulating

positive inotropy. However, as these differences were not all significant, a second study was undertaken to increase the sample size.

In contrast to study 1, data from study 2 demonstrated that baseline responses were not affected in the presence of ET antagonists. Furthermore, responses to exogenous ET-1 or increasing $[Ca^{2+}]_i$ were not modified by any treatment. However, a significant difference was reported between antagonist groups in response to isoprenaline, suggesting a major disparity between the two studies, which could not be fully explained by competitive antagonism or the complexity of receptor interaction. In addition, inactivity of the receptor antagonists was also ruled out as other *in vitro* experiments in our laboratory were achieving appropriate responses using the same batch of drug. An alternative explanation may be that the drugs were perhaps unable to penetrate the tissue, as suggested be the lack of modulation of responses to ET-1 by any antagonist or combination.

Animals of study 1 and study 2 were age matched and weight matched yet different contractile results were reported. One obvious difference between the two studies was the time point, study 1 was carried out 6 months prior to study 2 (January/February and July/August respectively). Therefore, semi-quantitative RT-PCR was carried out on the tissues from each study to establish if there was a molecular basis for the functional differences.

ANP and ET system mRNA expression was significantly elevated in myocardial samples from study 2 compared to study 1, despite the tissues being processed concurrently, with the same starting quantity of total RNA. Changes in mRNA levels do not necessarily reflect a change in protein level. However, in correlation with the contractile changes, it is possible that the seasonal variation in ET system mRNA expression was translated into protein. An increase in ET receptor level would have increased competitive antagonism between endogenous ET-1, BQ123 and BQ788. ppET-1 was also elevated in myocardial samples (and perhaps translated into mature ET-1), therefore, stretch of papillary muscles in the preparatory stages may have released more endogenous ET-1 to compete with antagonist for receptor binding

sites. It is not fully understood how or why laboratory animals would undergo a seasonal variation of the ET system as they are housed in climate-controlled conditions with a 12 hour light-dark cycle. However, it is not altogether unlikely, as other genes are known to vary in expression periodically (Kastrup & Christensen, 1984). Disparity between other factors many also have contributed to the differences between groups. It may be that conditions during carriage to the animal house were different, or that the stress levels experienced by the animals differed, possibly due to the animal handler or other activity in the animal house at the time. An observation that was noted was that the body weight of study group 2 increased more rapidly while in the animal house awaiting use, however, this was not considered significant at the time and was not investigated.

Therefore, in conclusion, data from this study suggests that ET receptors are possibly involved in regulating the contractile responses to ET-1 in isolated cardiac tissues, consistent with previous reports (Kasai *et al.*, 1994; Beyer *et al.*, 1996; Qi *et al.*, 2001). In addition, positive inotropic responses have also been shown to be mediated by both receptors in healthy human atrial and ventricular myocardial strips (Opgaard *et al.*, 2000). Binding studies have confirmed the 80:20 distribution of ET_A:ET_B receptors in rat ventricular myocardium (Qi *et al.*, 2001). In contrast, studies using isolated cardiomyocytes maintain that ET_A receptors are solely responsible for mediating the positive inotropic responses of ET-1 mainly through stimulation of Ca^{2+} channels (Kelso *et al.*, 2000). Others suggest that ET-1 exerts the positive inotropic effect mainly through the activation of the ET_A receptor-mediated Na^+/H^+ exchanger (Takeuchi *et al.*, 2001). Seasonal variation of the ET system has not previously been reported and it is not understood why it would undergo change. To confirm these findings, functional and molecular investigations would have to be repeated at one or both of the time points as well as performing binding studies on myocardial samples. However, there was not enough time available to investigate this phenomenon further during the period of this study. In addition, the use of antagonists in this study has also exposed the complexity of ET receptor interactions, with the possible indication of ET receptor crosstalk.

Chapter 5

Assessment of the LV end-systolic pressure-dimension relationship by transthoracic 2-dimensional echocardiography in rats

5.1 Introduction

Characterisation of the basic mechanical properties of ventricular contractility has typically been investigated at the myocardial level due to the complexity of assessing the instantaneous pressure and volume of the intact ventricle. Regulation of cardiac performance can be influenced by a variety of factors including heart rate, end-diastolic volume, arterial blood pressure and inotropic background, therefore evaluation of ventricular performance *in vivo* ideally requires load insensitive measures of both systolic and diastolic properties.

The Frank-Starling mechanism describes the intrinsic ability of the heart to increase contractile force in response to elevated end-diastolic volume. Using this principle, studies in the '70s, using isolated canine hearts found that end-systolic values from the pressure-volume relations, taken at isochronic points of heartbeats occurring under different conditions had a linear relationship. The inclination of this straight line was termed the end-systolic pressure-volume relationship (ESPVR) and provided a good representation of ventricular contractility (Suga *et al.*, 1973; Sagawa *et al.*, 1977). ESPVR is now regarded as an index of contractility and has been validated as a fundamental description of systolic cardiac mechanics (Mahler *et al.*, 1975; Maughan *et al.*, 1984).

Obstruction of the inferior vena cava by balloon catheter (Chen *et al.*, 1997), occlusion of the inferior vena cava (Sato *et al.*, 1998; Nemoto *et al.*, 2002) and aortic banding (Bregagnollo *et al.*, 2000) are experimental methods of adjusting the preload or afterload respectively in humans and animal models. However, the desired pressure changes can be achieved more accurately using vasoactive drugs and can generate a family of curves that go in both directions from baseline. Altering venous distribution with infusions of sodium nitroprusside (SNP) and phenylephrine (PE) has previously been demonstrated in mice to provide a linear ESPVR (Williams *et al.*, 1998). Administration of SNP, a direct endothelium-dependent vasodilator, reduces peripheral resistance, which reduces the afterload and shifts the pressure-volume loop (PV loop) down and to the left via the Frank-Starling mechanism. SNP is reported not to affect cardiac contractility (Crystal & Gurevicius, 1996). In

contrast, PE elicits vasoconstriction via α_1 adrenoceptors, increasing the afterload to shift the PV loop up and to the right, however, the effects of PE on myocardial contractility remain ambiguous (Kronenberg *et al.*, 1989; Strang & Moss, 1995).

Technological advancements such as the conductance catheter (Sato *et al.*, 1998), have provided a more accurate measurement of combined pressure and volume values, while two-dimensional transthoracic echocardiography allows a less invasive, repeatable assessment of LV dimension and volume. This requires dedicated and expensive equipment. Small animal high frequency echocardiographic probes are now available and are employed routinely to evaluate cardiac geometry and function of rats and mice *in vivo* (Litwin *et al.*, 1994; Williams *et al.*, 1998). Relatively inexpensive high fidelity pressure catheters are also available (Millar Instruments) and combining these techniques allows the assessment of the ESPVR. The assessment of ventricular contractility *in vivo* by this method has not previously been reported in the coronary artery ligated rat.

In Chapter 4, myocardial responses to endothelin-1 (ET-1) and endothelin antagonists failed to provide indication of a role for the ET_A and ET_B receptors in the heart. In this study, by assessing cardiac contractility *in vivo*, following chronic administration of the mixed $ET_{A/B}$ receptor antagonist bosentan, the role of endogenous ET-1 may become clearer.

The aims of this chapter were to assess cardiac contractility *in vivo* by investigating the end-diastolic pressure-dimension relationship of sham-operated and coronary artery ligated rats using an echocardiographic approach. In normal animals, following chronic treatment with the dual $ET_{A/B}$ receptor antagonist bosentan, possible contribution of endogenous endothelin to cardiac contractility was investigated.

5.2 Methods

Two-dimensional transthoracic echocardiography in conjunction with simultaneous LV pressure data from a Millar catheter was used to construct pressure-dimension

loops for the investigation of LV contractile function of (i) coronary artery ligated (CAL) compared to sham-operated rats and (ii) rats chronically treated with the $ET_{A/B}$ receptor antagonist bosentan compared to placebo.

5.2.1 The experimental groups

To evaluate the cardiac performance of failing and nonfailing animals, forty male Wistar rats were chosen at random to undergo CAL or sham-operated surgery as previously described in section 2.1.2. Subsequently, 12 weeks following surgery, the animals were weighed, anaesthetised and instrumented for haemodynamic and echocardiographic analysis (as described in section 2.1.3.2).

To investigate the influence of endogenous ET-1 on cardiac contractility, a group of healthy male Wistar rats (450-550g, Charles River, U.K.) were randomly assigned to either drug (dual $ET_{A/B}$ receptor antagonist bosentan, 100 $mg \cdot kg^{-1} \cdot day^{-1}$, n=6) or placebo group (n=6). Bosentan (25 $mg \cdot ml^{-1}$, Acetlion Pharmaceuticals Ltd., Switzerland) was freshly prepared daily as a microsuspension in 5% gum arabic (Sigma, see section 2.4) and administered by gastric gavage once daily for 7 days. The bosentan dose (100 $mg \cdot kg^{-1} \cdot day^{-1}$) was chosen as the maximally effective dose on the basis of *in vivo* pharmacological studies by Clozel *et al.*, (1994). After a week of treatment, animals were weighed, anaesthetised and haemodynamic and echocardiographic studies were performed as described before in section 2.1.3.2.1. Additionally, at the end of the PE infusion and a 20 minute stabilisation period, a bolus of bosentan (30 $mg \cdot kg^{-1}$) was infused into the femoral vein of the placebo animals to assess the acute effects of dual $ET_{A/B}$ blockade. The haemodynamic effects were observed and echocardiographic images were captured 30 minutes after acute bosentan administration.

Halothane was used for anaesthesia as an initial investigation showed that it controlled and maintained the level of anaesthesia more adequately than Sagatal (sodium pentobarbital). Initial studies also obtained the concentration and infusion rates of SNP and PE, which achieved the desired change of pressure.

5.2.2 Haemodynamic and echocardiographic data analysis

Haemodynamic parameters such as heart rate (HR), mean arterial pressure (MAP), LV end-systolic (LVSP) and end-diastolic pressures (LVEDP), maximal rate of pressure rise (dP/dt_{max}) or decline (dP/dt_{min}) were measured from the CHART data. Echocardiographic images were acquired digitally (Diasus, Dynamic Imaging) using a 10-22MHz probe. Files were transferred to a laptop computer (DELL, Inspiron 8000) and analysed in conjunction with the associated pressure data using Image Analyser software (version 1.1, ©David Anderson, Dynamic Imaging).

Two-dimensional long-axis echocardiographic images were viewed simultaneously with pressure data from the micromanometer. LV internal dimension values were obtained by manually tracing around the LV borders for at least three cardiac cycles, using Image Analyser software. LV pressure and internal dimension data was then exported to a spreadsheet. Initial investigation showed that the waveforms were slightly out of synchronicity (*Figure 5.1*). This was more obvious for cycles acquired later in the acquisition period. Therefore LV internal dimension and pressure values were averaged from the first cardiac cycle of at least three consecutive data sets of similar pressure, captured under each condition. The mean values were represented graphically by pressure-dimension loops (PD loops) using GraphPad Prism software (Version 3.02, GraphPad Software, Inc.). In the human, LV pressure measurements are typically combined with LV volume measurements to generate a pressure-volume loop (PV loop). LV area can be converted to LV volume by combining the value with the ventricular long-axis (area-length method, $V=A^2 \cdot 8.0 / (3.0 \cdot \pi \cdot L)$; where V, volume; A^2, area; L, ventricular long-axis; St. John Sutton *et al.*, 1996). However, due to the geometric assumption of the derivative and border distortions in some echo images, it was considered to be adding unnecessary error to the results and therefore, PD, rather than PV loops were derived.

End-systole was defined as the smallest LV internal dimension (ESD) and end-diastole defined as the onset portion of the upstroke of the PD loop, the largest LV internal dimension (EDD). In each animal, the LV end-systolic pressure (ESP) and ESD values were taken (the top left-hand corner of the PD loop) as co-ordinates (x,y)

for each condition (baseline, SNP, PE). Simple linear regression was used to fit a line through these points. The angular coefficient obtained, defined the value of the end-systolic pressure-dimension relation (ESPDR) for each animal.

LV systolic (LVSD) and diastolic (LVDD) diameters and ESD and EDD were measured at baseline with the aid of the Image Analyser software. LV fractional shortening (SF) was calculated from the equation: (LVDD - LVSD)/LVDD x 100 and LV ejection fraction (EF) from: (EDD - ESD)/EDD x 100.

Drug infusion of SNP or PE to achieve a desired pressure change was kept to a minimum volume by using concentrated solutions, low infusion rates and short duration of infusions, however, some volume loading still occurred as a consequence. To establish whether cardiac volume loading had a significant effect on LV compliance, the inclination of the diastolic portion of each PD loop was assessed and compared to the baseline value in each group.

Comparison between groups was performed by unpaired Student's *t*-test or one-way analysis of variance followed by Bonferroni's multiple comparison test where appropriate. A P value <0.05 was considered significant. Correlation analysis was used to investigate the interaction between the parameters measured. The Pearson correlation coefficient (r) was calculated for each simple linear regression, to determine the closeness of the straight line to the points.

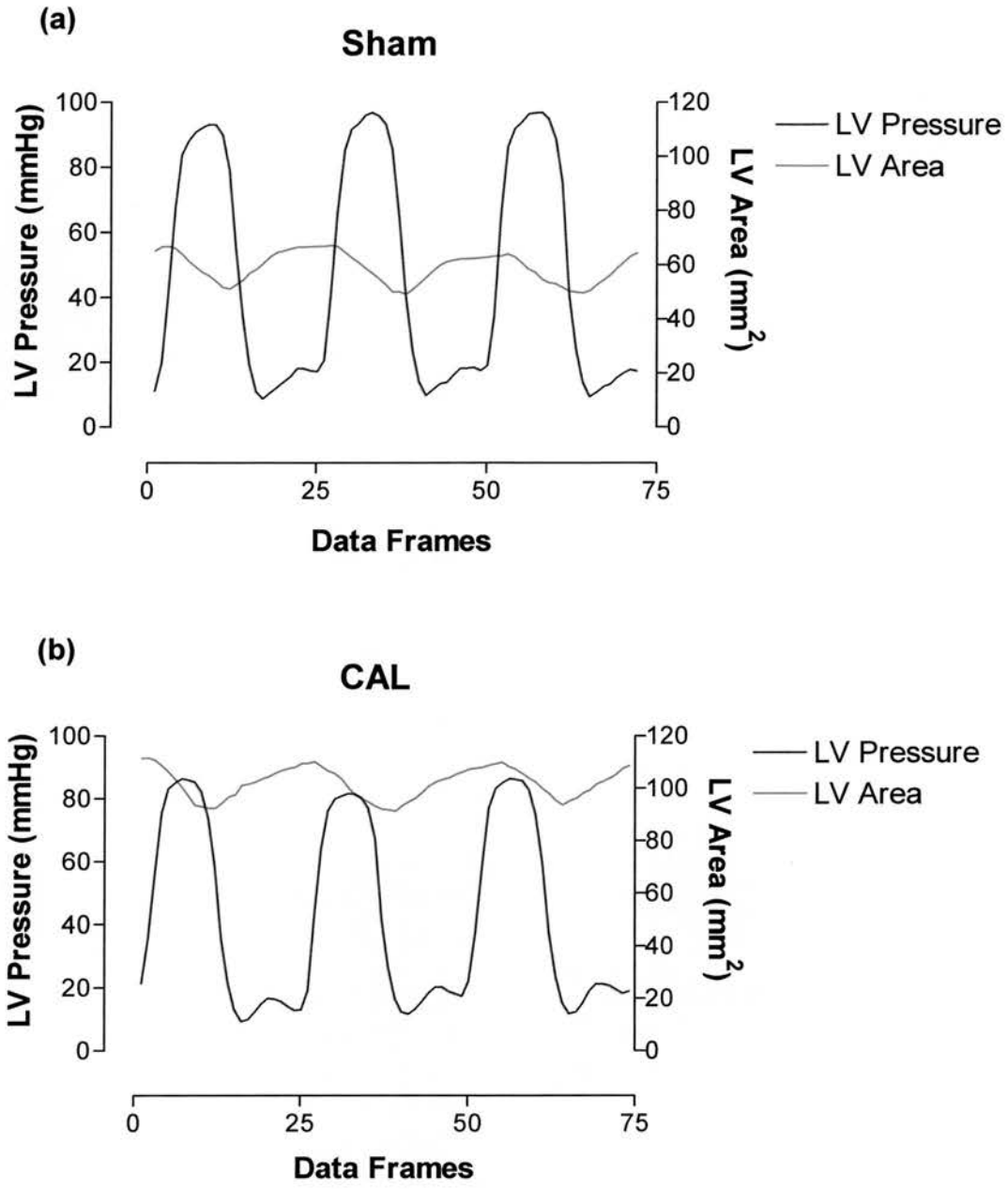


Figure 5.1 Examples of superimposed left ventricular pressure and dimension values from (a) sham-operated and (b) CAL rats, illustrating the progressive loss of synchronicity.

5.3 Results

The results from the two studies, CAL versus sham and bosentan versus placebo are reported separately in this section.

5.3.1 Effects of 12 week chronic CAL

5.3.1.1 Effects of CAL on mortality

Forty rats underwent CAL for the induction of CHF. Deaths occurring during or immediately following surgery were 50% of the whole group, due mainly to ischaemic arrhythmias. Only one CAL animal died in the 24hr period after surgery. No early or late deaths of sham-operated animals occurred.

5.3.1.2 Baseline haemodynamic and echocardiographic measurements

At 12 weeks post-ligation, cardiac haemodynamic parameters were recorded *in vivo* under general anaesthetic using a Millar catheter placed via the right carotid artery. CAL animals with LVEDPs ≥ 10mmHg were considered to have LV dysfunction (LVD), a significant decrease in $dP/dt_{max}/P$ and $dP/dt_{min}/P$ was also observed, however, no significant difference was obtained in HR, MAP, dP/dt_{max} or dP/dt_{min} between groups (*Table 5.1*). LV EF and SF were both significantly reduced in the CAL group compared to the sham group (*Table 5.1*). In *Figure 5.1* it can be seen that the LV dimensions were greater in the infarcted rat compared to the sham-operated rat. This individual CAL rat had a huge transmural infarct (68% of the free LV wall), a large heart: body weight ratio (3.86 $g \cdot kg^{-1}$) and large internal dimensions (see *Figure 5.1*, compare LV area of sham and CAL). However, not all CAL animals had such substantial infarcts (31.9 ± 9.3%, n=5) or heart: body weight ratio (sham, 2.77 ± 0.13 $g \cdot kg^{-1}$, n=5; CAL, 3.02 ± 0.22 $g \cdot kg^{-1}$, n=6) and this was also reflected in the averaged LV internal diastolic dimensions (*Table 5.1*).

There was no significant correlation between LVEDP, EF or SF in each group. Although LVEDP values tended to be inversely associated the EF in the CAL group,

where higher LVEDPs were associated with lower EF values, the condition of linearity was not met (r = 0.15). A stronger correlation was observed between EF and SF, where the greater the EF, the greater the associated SF but again, linearity was not achieved for the relationship (sham, r = 0.54: CAL, r = 0.76). Additionally, a trend was observed between EF and dP/dt_{max} where a greater dP/dt_{max} was associated with a greater EF, however, correlation analysis was not significant (sham, r = 0.60: CAL, r = 0.67).

5.3.1.3 The end-systolic pressure-dimension relationship of sham-operated or CAL rats

All rats showed the expected shifts of PD loops for SNP or PE infusions. *Figure 5.2* illustrates the PD loops obtained from individual animals under the different afterload conditions. For each animal, simple linear regression was used to analyse the end-systolic points of baseline, SNP and PE conditions to determine the ESPDR. Obtaining the ESPDR index assumes the existence of linearity between the end-systolic points used for determining each of the straight lines. In this study the Pearson correlation coefficient values (r) ranged from 0.95 to 0.99 for sham and 0.90 to 0.99 for CAL respectively, indicating that the condition of linearity was met in most cases (*Table 5.2*). Statistical analysis showed that ESPDR values were significantly decreased in the CAL group compared to the sham group and that the *y*-intercept had also significantly shifted, indicating a significant reduction in contractility (*Table 5.2*). No significant association was identified between ESPDR, LVEDP, EF or SF values.

Linear regression analysis was used to determine whether volume loading had an effect on the SNP or PE PD loops. The slope of the diastolic portion of the SNP or PE PD loops were not significantly different from the baseline loop in either the sham or CAL group (sham: baseline, 0.46 ± 0.08 mmHg/mm^2; SNP, 0.32 ± 0.07 mmHg/mm^2; PE, 0.79 ± 0.04 mmHg/mm^2; CAL: baseline, 0.48 ± 0.06 mmHg/mm^2; SNP, 0.19 ± 0.08 mmHg/mm^2; PE, 0.74 ± 0.16 mmHg/mm^2), in the majority of cases, the condition of linearity was met. *Figure 5.3* illustrates this effect in an individual sham and individual CAL animal.

Table 5.1. Baseline haemodynamic and echocardiographic characteristics of sham-operated and CAL animals indicating LV dysfunction.

	Sham (n=5)	CAL (n=8)
Body Weight (g)	518 ± 3.3	520 ± 27.6
Heart: body weight ratio ($g \cdot kg^{-1}$)	2.77 ± 0.13	3.02 ± 0.22
HR (bpm)	316.4 ± 6.4	321 ± 11.4
MAP (mmHg)	78.9 ± 1.8	78.2 ± 7.1
LVEDP (mmHg)	5.7 ± 1.0	15.5 ± 1.5***
dP/dt_{max} ($mmHg \cdot ms^{-1}$)	3550.7 ± 182.8	3595.2 ± 203.2
$dP/dt_{max}/P$	62.2 ± 2.1	54.6 ± 1.88*
dP/dt_{min} ($mmHg \cdot ms^{-1}$)	-4251.1 ± 420.4	-3828.6 ± 245.4
$dP/dt_{min}/P$	-71.1 ± 3.8	-55.7 ± 2.8**
LV diastolic dimension (mm^2)	80.3 ± 8.6	82.93 ± 4.8
LV Ejection Fraction (%)	31.1 ± 2.6	22.4 ± 1.2**
LV Shortening Fraction (%)	18.7 ± 1.9	13.6 ± 1.3*

Values are means ± SEM, *P<0.05, **P<0.01, ***P<0.001 compared to sham. HR, heart rate; MAP, mean arterial pressure; LVEDP, left ventricular end-diastolic pressure; dP/dt_{max}, maximal rate of LV pressure rise; dP/dt_{min}, maximal rate of LV pressure decline.

Table 5.2. Values of left ventricular end-systolic pressure-dimension relation (ESPDR) obtained from sham and CAL rats. The y-intercept and the correlation coefficients (r) of the straight lines determining the ESPDR are included.

	Sham (n=4)			CAL (n=4)		
	ESPDR ($mmHg/mm^2$)	y-intercept (mmHg)	r	ESPDR ($mmHg/mm^2$)	y-intercept (mmHg)	r
	6.01	-397.0	0.99	0.78	49.1	0.99
	6.39	-221.1	0.95	3.18	-19.8	0.94
	5.06	-135.4	0.99	2.17	-127.7	0.99
	8.90	-371.5	0.99	1.73	-15.7	0.90
Mean	6.59	-281.25	-	1.96**	-28.5*	-
SEM	0.82	62.2	-	0.50	36.6	-

*P<0.05, **P<0.01 compared to sham.

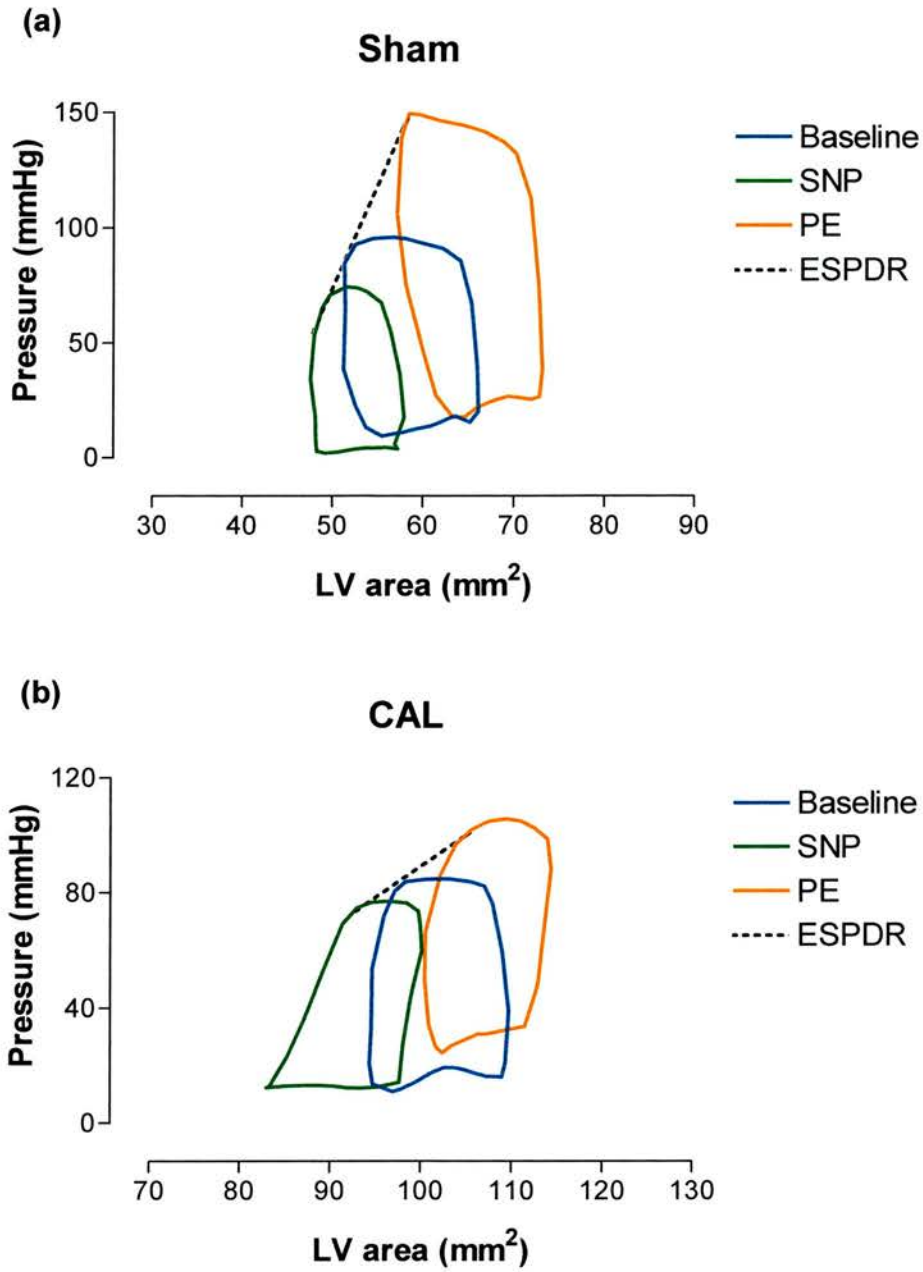


Figure 5.2. Examples of pressure-dimension loops from individual experiments (a) sham-operated or (b) CAL rats under baseline conditions and during the infusion of sodium nitroprusside (SNP, bottom left loop) or phenylephrine (PE, top right loop). The end-systolic pressure-dimension relationship (ESPDR) is also indicated for each animal.

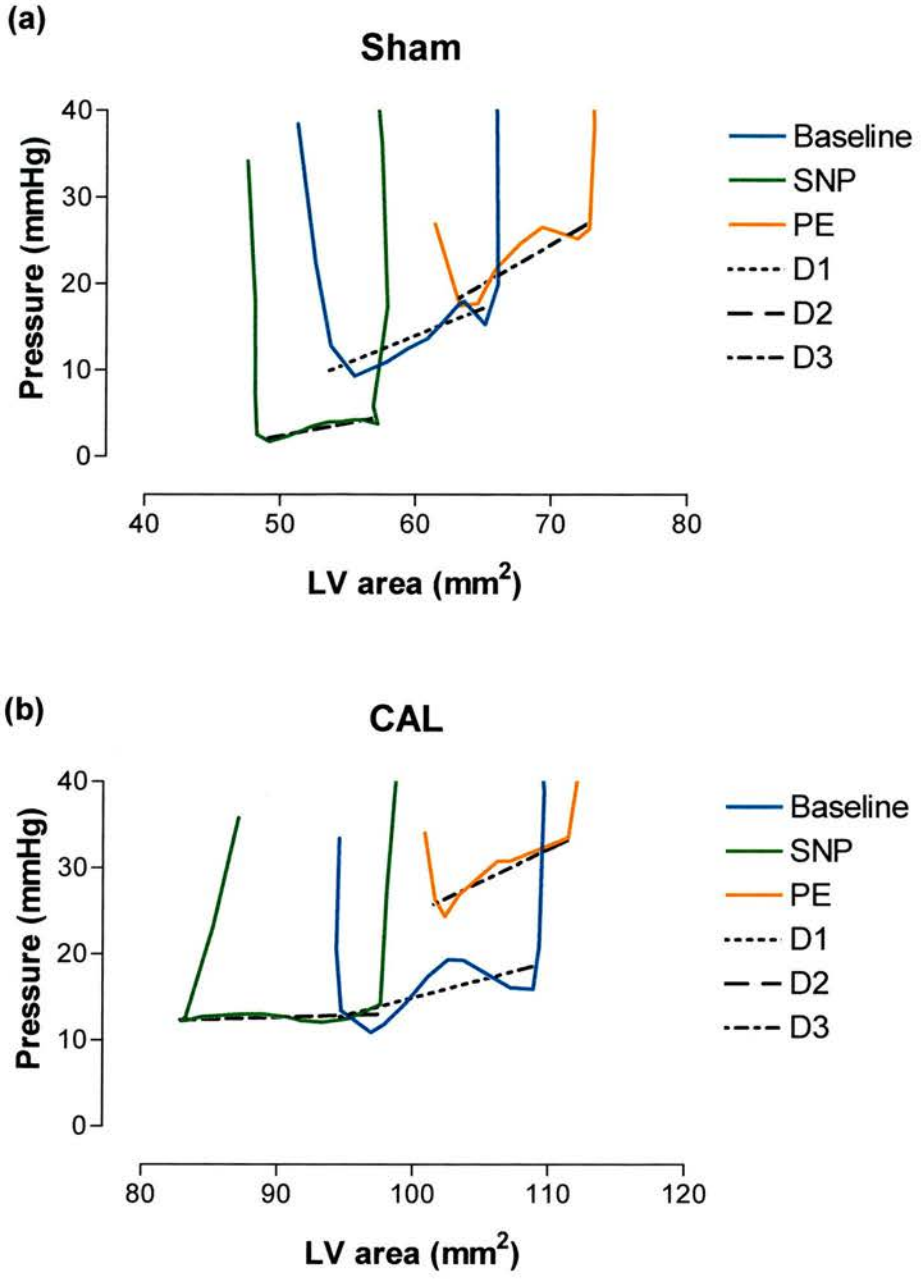


Figure 5.3. Examples of the diastolic phase of the pressure-dimension loops from (a) sham and (b) CAL rats to illustrate that infusion volumes do not significantly alter the diastolic slope compared to baseline. Linear correlation analysis of the diastolic portion of each PD loop is represented, where: D1, baseline; D2, SNP; D3, PE.

5.3.2 Effects of chronic administration of bosentan

5.3.2.1 Baseline haemodynamic and echocardiographic measurements

Haemodynamic parameters were not significantly altered in response to 1 week chronic treatment with the dual $ET_{A/B}$ receptor antagonist bosentan in normal animals (*Table 5.3*). No change was observed in the basal HR, MAP, LVEDP or differential functions of the pressure waveform. LV EF and SF were not significantly different between groups (*Table 5.3*). Pressure and dimension averages were calculated as before from at least three first cardiac cycles of multiple data sets captured under each preload condition. No significant difference was observed in LV internal diastolic dimensions between groups (*Table 5.3*).

5.3.2.2 End-systolic pressure-dimension relationship of placebo or bosentan treated rats

Infusions of SNP and PE produced the expected shifts of the PD loop in each animal, however, no significant change occurred in the ESPDR as a result of chronic bosentan administration (*Figure 5.4*). The angular coefficient and the *y*-intercept values for each group were not significantly altered (*Table 5.4*). The condition of linearity was met in the majority of cases and the correlation coefficient values (r) ranged from 0.78 to 0.99 for the placebo group and 0.95 to 0.99 for the bosentan treated animals (*Table 5.4*). In addition, it was observed that the ESPDR values for healthy animals treated with placebo or bosentan differed significantly from the sham-operated group of the previous study (sham 6.59 ± 0.82mmHg/mm^2; placebo, 2.93 ± 0.29 mmHg/mm^2, $P<0.01$ compared to sham; bosentan, 2.88 ± 0.19mmHg/mm^2, $P<0.001$ compared to sham).

The diastolic portions of the SNP and PE loops were compared to baseline using simple linear regression analysis. The angular coefficient of the SNP or PE diastolic parts were not significantly different from baseline for the placebo or the bosentan group (placebo: baseline, 0.67 ± 0.21mmHg/mm^2; SNP, 0.11 ± 0.13mmHg/mm^2; PE, 0.84 ± 0.16mmHg/mm^2; bosentan: baseline, 0.64 ± 0.08mmHg/mm^2; SNP, 0.33 ±

0.15mmHg/mm^2; PE, 1.07 ± 0.34mmHg/mm^2). Linearity was obtained in the majority of cases.

5.3.2.3 Effect of acute administration of bosentan

After a stabilisation period following the ESPDR investigation, a bolus of the $ET_{A/B}$ antagonist bosentan (30 mg·kg^{-1}, i.v.) was infused into placebo animals. Over the subsequent 40-minute period, a triphasic change in LV pressure was observed. Acute administration of bosentan resulted in a significant decrease in peak LVSP (117.4 ± 6.2 to 99.4 ± 3.5mmHg, n=6, P<0.05) 10 minutes post-infusion. Subsequently, 16-18 minutes after infusion, the pressure increased (110.1 ± 1.9mmHg), before reducing again, 30 minutes post-infusion (95.9 ± 4.7mmHg, n=6, P<0.05 compared to pre infusion). The systolic pressure continued to fall progressively thereafter. MAP was significantly reduced compared to pre-infusion levels (95.99 ± 4.48 to 61.30 ± 2.23 mmHg, n=6, P<0.001). The PD loops plotted from data captured 32 - 36 minutes post-infusion were shifted down and to the left, with a similar value for the ESPDR slope to that obtained with SNP (*Figure 5.4*).

Table 5.3. The effect of chronic administration of bosentan, a dual $ET_{A/B}$ antagonist, on baseline haemodynamic and echocardiographic parameters.

	Placebo (n=6)	Bosentan (n=6)
Body Weight (g)	508 ± 12	484 ± 17
Heart: body weight ratio ($g \cdot kg^{-1}$)	2.78 ± 0.09	2.68 ± 0.10
HR (bpm)	359.9 ± 15.9	351.4 ± 15.8
MAP (mmHg)	97.0 ± 3.6	94.7 ± 2.7
LVEDP (mmHg)	4.6 ± 0.9	5.1 ± 1.3
dP/dt_{max} (mmHg/ms)	4040.3 ± 448.8	4124.3 ± 297.4
$dP/dt_{max}/P$	70.5 ± 5.3	66.4 ± 3.2
dP/dt_{min} (mmHg/ms)	-4304.0 ± 495.8	-4604.7 ± 484.2
$dP/dt_{min}/P$	-74.3 ± 7.6	-72.3 ± 5.9
LV diastolic dimension (mm^2)	78.1 ± 4.2	72.3 ± 5.1
LV Ejection Fraction (%)	34.3 ± 1.8	28.9 ± 2.0
LV Shortening Fraction (%)	28.6 ± 2.3	23.3 ± 1.7

Values are means ± SEM. HR, heart rate; MAP, mean arterial pressure; LVEDP, left ventricular end-diastolic pressure; dP/dt_{max}, maximal rate of LV pressure rise; dP/dt_{min}, maximal rate of LV pressure decline.

Table 5.4. Left ventricular end-systolic pressure-dimension relationship (ESPDR) values obtained from placebo and bosentan treated rats. The y-intercept and the correlation coefficient (r) values of the ESPDR straight lines are included.

	Placebo (n=6)			Bosentan (n=6)		
	ESPDR ($mmHg/mm^2$)	y-intercept (mmHg)	r	ESPDR ($mmHg/mm^2$)	y-intercept (mmHg)	r
	1.77	7.6	0.99	2.80	-53.4	0.99
	3.75	-63.8	0.99	3.61	-66.6	0.97
	2.84	-63.5	0.98	2.38	-42.1	0.99
	2.56	-51.3	0.78	3.25	-63.7	0.99
	3.26	-75.2	0.97	2.51	-67.7	0.91
	3.39	-144.6	0.89	2.71	-80.5	0.95
Mean	2.93	-65.1	-	2.88	-62.3	-
SEM	0.29	19.9	-	0.19	5.37	-

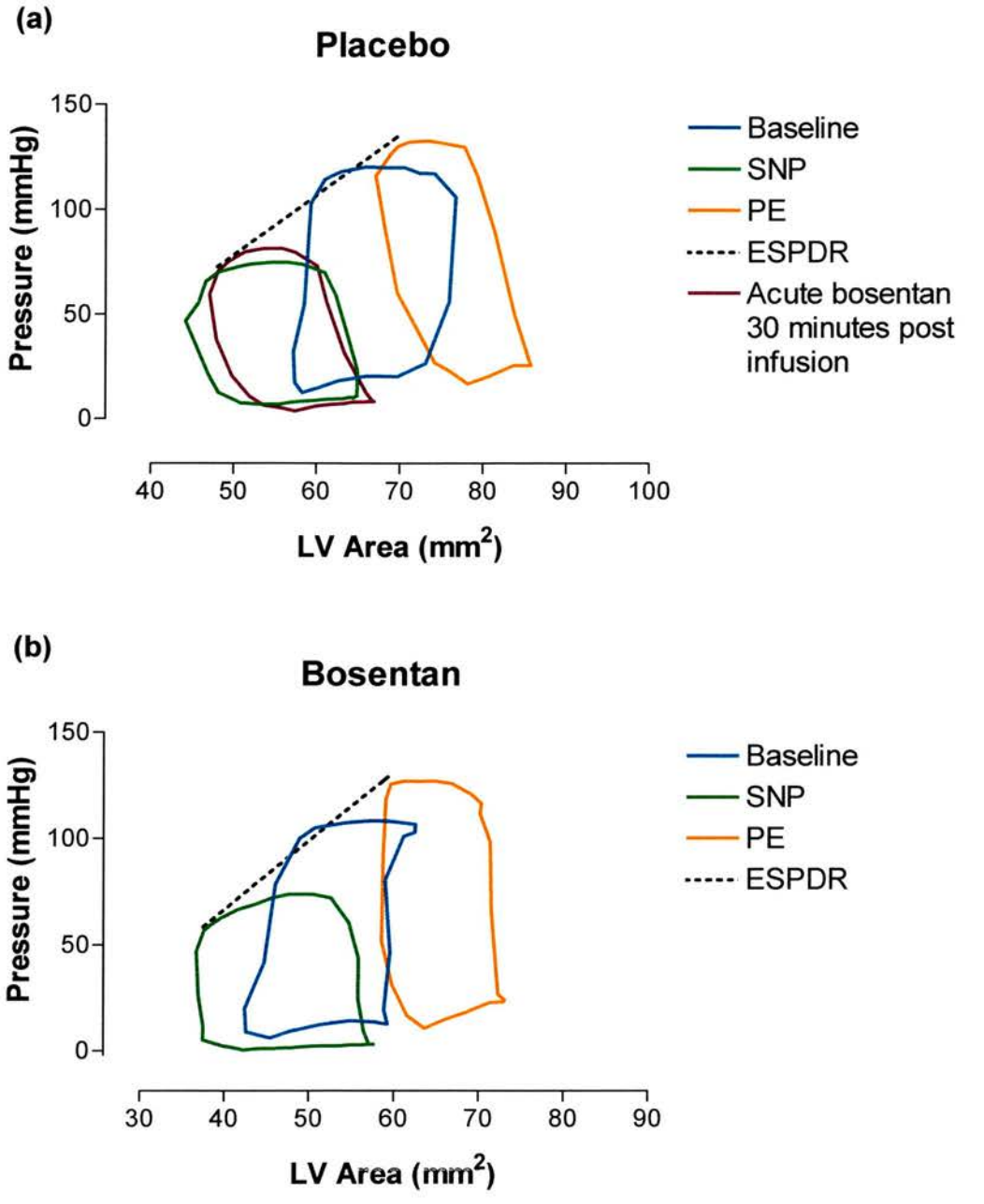


Figure 5.4. Examples of the pressure-dimension loops from individual experiments (a) placebo or (b) chronic effects of bosentan treatment, under baseline conditions and during the infusion of sodium nitroprusside (SNP, bottom left loop) or phenylephrine (PE, top right loop) in control rats. The end-systolic pressure-dimension relationship (ESPDR) is indicated for each animal. The effect of acute bosentan administration on the PD loop of the placebo animal is also included.

5.4 Discussion

The purpose of this study was to assess the cardiac contractility of rats *in vivo* using the ESPDR index of contractile state. Ventricular contractility was evaluated in rats 12 weeks following CAL surgery and compared with sham-operated animals to determine the effect of experimental heart failure. In addition, normal rats were given bosentan or placebo for one week to examine the contribution of endogenous ET-1 to ventricular contractility.

5.4.1 Pressure-dimension relation in infarcted rat hearts

As previously described, deterioration of haemodynamic parameters are characteristic of LV dysfunction in the chronically infarcted rat heart (Pfeffer *et al.*, 1979). In this study, a significant elevation of LVEDP and reduced differential functions of the LV pressure waveform were observed in rats 12 weeks following CAL surgery, indicating myocardial dysfunction. These findings were supported by a significant reduction in LV EF and SF as evidenced by echocardiographic assessment of LV geometry (Litwin *et al.*, 1994). All groups showed the expected shift of the PD loops for SNP and PE infusions, indicating that different afterload states were achieved. The main findings of this investigation were that ESPDR values were significantly reduced in CAL compared to sham-operated rats, further substantiating the reduction of ventricular contractility.

Histological assessment of hearts showed that not all CAL rats had developed transmural scars, indicating variability in the degree of dysfunction sustained. However, within the CAL group, linear correlation analysis between parameters was inconsistent, as the rats with larger infarcts did not necessarily display the highest LVEDP or the lowest EF, SF or ESPDR values. Therefore, an accurate interpretation of the ESPDR as an index of cardiac contractility *in vivo* in heart failure was restricted as rats exhibited varying degrees of LV dysfunction. Using a larger sample size and imposing a condition which would exclude rats with infarcts under a specified size, as many studies do (Bauersachs *et al.*, 2000), may provide a more accurate correlation.

5.4.2 Effects of chronic administration of bosentan on cardiac haemodynamics

ET-1 is the most potent vasoconstrictor substance known and contributes to the increased peripheral resistance in humans and rats with chronic cardiac dysfunction by inducing peripheral vasoconstriction (Kiowski *et al.*, 1995; Teerlink *et al.*, 1994). ET-1 has also previously been shown to exert positive inotropic effects in isolated cardiac tissue preparations (see Chapter 3) but isolated heart and *in vivo* studies have failed to provide consistent evidence for the role of ET-1 in regulating myocardial function. In this study the $ET_{A/B}$ receptor antagonist bosentan was administered to healthy adult male rats for one week to evaluate the role of endogenous ET in normal cardiac function. As ET-1 is known for its potent vasoconstrictor properties, it would be logical to assume that dual receptor blockade would induce dilation. However, in the current chronic study, treatment did not affect any of the haemodynamic parameters measured such as MAP, LVSP or dP/dt_{max}. Similar responses have been reported in previous studies where sham-operated animals were administered bosentan for 8 weeks (Fraccarollo *et al.*, 1997) or 11 weeks (Bauersachs *et al.*, 2000) following surgery. However, bosentan treatment was reported to affect the parameters of the infarcted animals in both studies (Fraccarollo *et al.*, 1997; Bauersachs *et al.*, 2000), indicating a role for ET-1 in pathological conditions.

Echocardiographic assessment of the LV showed that there was no difference between treatment groups for the EF or SF, suggesting that ventricular function had not been altered by ET receptor blockade. Baseline PD loops were not significantly different between groups, suggesting that bosentan had not altered the peripheral resistance to change the afterload. Infusion of SNP and PE produced the expected PD loop shifts in both placebo and bosentan groups. The condition of linearity was met for the ESPDR, however, ESPDR values were not significantly different between groups and neither was the *y*-intercept value altered, suggesting that contractility was unchanged between groups. These findings suggest that endogenous ET-1 does not play a central role in regulating cardiac function *in vivo* in the healthy rat. Lack of responses in normal animals, as evidenced by the unaltered contractile parameters, is possibly due in part to the reduction of nitric oxide via endothelial ET_B receptor

blockade. However, the most likely explanation is that other mechanisms, such as the renin aldosterone angiotensin system, independent of ET-1 and ET receptors, become involved and compensate for the influence of ET-1, therefore maintaining normal haemodynamic pressures to conserve contractile function.

5.4.3 Effects of acute administration of bosentan on cardiac haemodynamics

Acute administration of bosentan induced a triphasic blood pressure response, resulting in a significant reduction of systolic and MAP, 30 minutes after infusion. The initial reduction (10 minutes post-infusion) was due to blockade of the constrictor $ET_{A/B}$ receptors, lowering peripheral resistance. This was then followed (16-18 minutes post-infusion) by a rise in pressure, possibly induced by an increase of plasma ET-1 levels, resulting from inhibition of ET_B receptor clearance and therefore, marked increase in competition for binding sites. The final reduction of LVSP indicated the ability of the acute administration of antagonist to exert its effects. Short-term reduction of MAP in response to acute administration of bosentan has previously been demonstrated in sham-operated rats using telemetry (Teerlink *et al.*, 1994). Two weeks after surgery, CAL and sham-operated rats with telemetry implants were administered a dose of either placebo or bosentan (100 $mg \cdot kg^{-1}$) and haemodynamics responses were recorded for 48 hours thereafter. The antagonist caused a reduction of MAP in the sham group for several hours after infusion, before returning to normal levels (Teerlink *et al.*, 1994). The triphasic response observed in the current study was not reported by Teerlink *et al.* (1994) because the telemetry data was averaged hourly, however, the response does support the current observations of the acute study.

PD loops captured 30 minutes after acute infusion of bosentan were shifted down and to the left, similar to those achieved by SNP, indicating a reduction of afterload. In most cases, the end-systolic points of the bosentan PD loops were close to the straight line of the ESPDR, suggesting that cardiac contractility was not altered by acute $ET_{A/B}$ receptor blockade. Direct assessment of the ESPDR by infusion of SNP and PE in the presence of acute bosentan was not possible due to the extended

duration of the experiment, therefore the cardiac responses to acute bosentan were not confirmed. However, a recent *in vitro* study demonstrated that endogenous ET-1 contributes to the Frank-Starling mechanism in isolated perfused hearts from hypertensive double transgenic rats (harbouring human renin and angiotensinogen genes) but not normotensive nontransgenic rats (Piuhola *et al.*, 2003). The study observed that bosentan impaired the Frank-Starling response in the hypertrophied rat hearts by affecting the systolic performance. Absence of this response in the normotensive nontransgenic animals agrees with the findings from the current investigation, implying that endogenous ET-1 only contributes to the maintenance of cardiac function in pathological conditions and indicating that the acute effects were due to the influence of ET-1 on the peripheral vasculature.

5.4.4 Effects of pericardiectomy on cardiac performance

Previous studies have demonstrated that removal of the pericardium in experimental models results in an increase of the maximal oxygen consumption, the maximal cardiac output and the maximal stroke volume, without changing the heart rate (Stray-Gundersen *et al.*, 1986; Hammond *et al.*, 1992). Comparison of the two study groups revealed that the ESPDR values were significantly greater for the sham-operated group compared to the placebo or bosentan groups, which were investigated in healthy animals. The effects of pericardiectomy are the most likely explanation why the contractility of the sham group was greater than that of healthy animals after placebo of bosentan treatment. In addition, the effects of pericardiectomy are also associated with increased LV end-diastolic dimension and an estimated increase in end-diastolic volume (Hammond *et al.*, 1992), which may explain why significant differences were not observed between the CAL and sham-operated groups of the current study.

5.4.5 Basal ET-1 activity in the normal heart

Positron emission tomography (PET) is a sensitive technique that has the power to quantify receptor-bound radioligands *in vivo*. In a recent study, the distribution of a radiolabelled dual $ET_{A/B}$ antagonist (^{11}C-L-753,037) was studied for its *in vivo*

kinetics, biodistribution and ET receptor binding characteristics in mice (Aleksic *et al.*, 2001). Results from the kinetic studies showed highest tracer uptake 5 minutes after injection in the liver (25%), moderate uptake levels in the kidneys (18.7%) and lungs (15.2%) and lowest levels in the heart (5.6%). However, the initial high levels in the liver, lungs and kidneys were followed by rapid washout over the next 10 minutes and a very slow clearance during the study period (2 hours after injection). In contrast, the level of radioactivity in the heart remained constant during the observation period, indicating that cardiac ET receptors exhibit unique binding characteristic *in vivo*. Johnström *et al.* (2002) reported a different pattern of binding characteristics in the rat using radiolabelled ET-1 (^{18}F-ET-1), where high levels of uptake were found in the lungs and kidney and moderate levels of uptake in the liver. In contrast with the Aleksic *et al.* (2001) study, distribution of binding was not reported in the heart (Johnström *et al.*, 2002), suggesting that binding was either below detection level or that the tracer was unable to bind to cardiac receptors. Considering the pharmacology and the other literature (Piuhola *et al.*, 2003), it is possible that high levels of ET-1 are not achieved in the normal heart and this concurs with the current findings, which suggest that endogenous ET-1 does not have a central role in regulating the function of the healthy myocardium.

5.4.6 Study limitations

Echocardiography has emerged as a useful noninvasive tool for the investigation of cardiovascular research in humans, rats and mice and has previously been shown to reliably evaluate LV dimensions in the intact heart (Litwin *et al.*, 1994; Williams *et al.*, 1998; Chen *et al.*, 1997; Urheim *et al.*, 2002). However, the evaluation of ventricular dimensions critically depends on image quality to correctly define the endocardial borders and it is accepted that echocardiographic images are harder to obtain in animals undergoing cardiac catheterisation due to incomplete border detection, particularly around the mitral-annular plane. This was one of the reasons why a number of the animals were excluded from the present study.

Automated digital echocardiography quantification (DEQ) has been carried out on cardiac patients and compared with simultaneous data from a conductance catheter to

evaluate the accuracy of the technique (Chen *et al.*, 1997). The study concluded that generally the two methods correlated well when the data was used to construct steady-state PV-loops but that stable DEQ images were more difficult to define during extreme loading transients. This is possibly due to viscoelastic myocardial accommodations as previous studies have identified that marked pressure (LVSP>200mmHg) or volume (LVEDP>25mmHg) overloads are out-with the physiological limits and result in deviations of LV PV relations in dogs (Le-Winter *et al.*, 1979; Way *et al.*, 1986). However, assessment of ESPDR behaviour during moderate afterload elevations in isolated (Maughan *et al.*, 1984) or *in situ* (Spratt *et al.*, 1987; Bregagnollo *et al.*, 2000) canine hearts, have concluded that the linearity of this contractility index does not significantly change during moderate pressure elevations. In the current study, variations in cardiac load did not reach the values reported (Le-Winter *et al.*, 1979) and therefore were not considered capable of triggering significant changes in the PD relations.

One of the major concerns associated with using an infusion method to alter afterload is the risk of cardiac volume loading, which is known to affect ventricular compliance. Previous studies have shown an increased gradient of the diastolic portion of the curve during volume loading with saline (Urheim *et al.* 2002). In the current study, diastolic inclinations were not significantly different between conditions, indicating that volume loading by i.v. infusion was not significantly evident in the SNP or PE protocols. This suggests that minimal changes in blood volume were accommodated and did not influence intrinsic cardiac inotropism to modify the ESPDR values. These findings indicate that the infusion method was suitable for the purposes of this study and confirm, within reasonable limits of error, that the contractility index provided a reliable measurement of ventricular contractility.

In conclusion, it has been demonstrated that echocardiographic assessment of the ESPDR during alteration of cardiac afterload is a reliable method of measuring cardiac contractility *in vivo* in the rat. Furthermore, reduced cardiac function of CAL rats as evidenced by elevated LVEDP, reduced EF, SF and differential waveforms of

LV pressure, were substantiated by the significant reduction of the ESPDR. In addition, in accordance with recent data, it has been shown that endogenous ET-1 does not appear to play a central role in cardiac contractility.

Chapter 6

Differential gene expression across the LV wall in failing human hearts

6.1 Introduction

Plasma levels of the vasoactive peptide endothelin-1 (ET-1) are found to be increased in patients with heart failure, irrespective of aetiology (McMurray *et al.*, 1992) and correlate with prognosis and disease progression (Pacher *et al.*, 1996). These observations suggest a pathophysiological role for the ET system in chronic heart failure. However, only scarce information is available on the activity and modifications of the cardiac ET-1 system in heart failure. Results from previous investigations have been inconsistent, possibly due to the type or severity of heart failure studied, the combination of treatments prior to surgery, or the portion of tissue investigated. ET-1 binding studies carried out by Pönicke *et al.* (1998) confirmed the co-existence of ET_A and ET_B receptors in both failing and non-failing human left ventricular (LV) myocardium and showed that the density of the ET_A receptor had the tendency to be increased in failing compared to non-failing human myocardial tissue. Similarly, Pieske *et al.* (1999) indicated an increase of ET_A receptors in LV tissue from end-stage failing hearts (dilated cardiomyopathy, DCM), with no change in ET_B receptor density. In contrast, Zolk *et al.* (1999) reported no significant difference of ET_A receptor mRNA or ET-1 mRNA in DCM tissue compared to non-failing LV tissue. However, the study did observe a significant decrease of ET_B receptor mRNA in DCM samples compared to non-failing, possibly explaining the elevated plasma ET-1 levels via reduced clearance.

The fundamental principles underlying ventricular mechanics in both physiological and pathological states requires an understanding of the relationship between the stresses and deformations acting on the muscle comprising the wall. In the human heart, wall stress is highest in the endocardial layer, decreasing towards the epicardium and this is partly attributed to the varying elastic stiffness of the wall material (Yin 1981). In addition, in diseases characterised by abnormal loading of the heart, normalisation of wall stress is thought to be the feedback signal that governs the rate and extent of ventricular hypertrophy. To date, transmural expression of the ET system in heart failure has not been assessed and as discussed above, no clear or consistent pattern of ET system expression in heart failure has emerged. Therefore, investigating different LV portions of hearts exhibiting different types of heart failure

may lead to clarification of the pathological alterations and may reveal whether ET system expression relates to wall stress.

Part of the force responsible for restoring resting length after contraction is provided by the elastic properties of titin (Helmes *et al.*, 1996). Titin, (also known as connectin), is the third most abundant sarcomeric filament system and is an integral part of the myofibril (see section 1.1.2). It is the largest protein known to date (3-4MDa) and is composed of over 27,000 amino acids (Labeit & Kolmerer, 1995). Single titin molecules extend across half the sarcomere, with their amino- and carboxy-termini crossing the Z-line and M-line respectively. The polypeptide is extensively modular and appears as a rod with beaded substructure, this is because it is mainly composed of fibronectin type 3 (FN3) and immunoglobulin (Ig) repeats, which fold into globular domains (Labeit *et al.*, 1992). The molecular segment confined to the I-band is a specialised segment that contains multiple Ig-like domains and a unique sequence rich in proline, glutamate, valine and lysine residues termed the PEVK segment (*Figure 1.1*). These domains reversibly unfold and can be viewed as 2 springs connected in series. The I-band of titin is functionally elastic and is responsible for passive tension generation upon stretch of non-activated striated muscle (Erickson, 1997; Linke & Granzier, 1998). In contrast, the titin segment that spans the A-band is functionally inelastic under physiological conditions. This is due to its strong lateral association with the myosin filaments (Itoh *et al.*, 1988; Labeit *et al.*, 1992; Soteriou *et al.*, 1993).

Different mobilities of skeletal and cardiac titins on SDS-gels have identified that titin is expressed in different isoforms in various tissues. These occur as a result of a series of alternative splicing events of I-band titin in different muscle (Labeit & Kolmerer, 1995). Differentially expressed Ig-repeats and PEVK domains in skeletal muscle titin produce a segment termed the N2A region, whereas cardiac muscle titin coexpresses two distinct sequenced variants, N2A and N2B, at sarcomere level (Labeit & Kolmerer, 1995; Linke *et al.*, 1996) (*Figure 1.1*). Labeit *et al.* (1997) previously suggested that expression of different tandem I-g segment lengths in various muscle types might be important for setting the physiological slack length,

whereas differential splicing of the PEVK-rich sequences may control the characteristic stiffness of relaxed muscle tissue. Indeed, N2B titin is shorter and has stiffer elastic properties than N2BA titin. The N2B region contains several Ig domains and sequence insertions unique to this isoform, while in comparison, N2BA titin, which contains both N2B and N2A elements, is larger and more compliant. It contains a much longer PEVK segment than N2B titin (Freiburg *et al.*, 2000) and contains an additional tandem Ig segment. Investigation of sarcomere length-force relations has identified that the stiffness of the myocardium is linked to the ratio of these titin domains and recent studies have also demonstrated that the ratio of cardiac isoforms differs between species (Cazorla *et al.*, 2000). In addition, Cazorla *et al.* (2000) also indicated that the expression ratio differed between the different layers of the porcine LV wall. Therefore, normal cardiac function and integrity of the myofibril is influenced by the extensibility of titin molecules via isoform expression.

Previous studies have demonstrated that major cardiomyocyte cytoskeletal proteins including titin, β-tubulin and desmin are differently regulated in cardiac hypertrophy (Collins *et al.*, 1996). Immunohistochemistry studies initially reported that titin was reduced and frequently disorganised in diseased human cardiac tissue (Hein *et al.*, 1994). Morano *et al.* (1994) also concluded that titin was reduced in the failing human myocardium but identified the expression of an additional protein band of lower molecular weight, now recognised as N2B titin. Therefore, it appears likely that instead of titin loss there is a switch in the isoform ratio, altered towards the stiffer element in pathological conditions. Abnormalities of titin expression in heart failure may be especially important because titin significantly influences sarcomeric elastic behaviour and is also necessary as a template for the organisation of newly synthesised myosin and actin filaments. To date, expression of titin isoforms has not been investigated across the LV wall. Transmural assessment may therefore establish whether the isoforms correlate to increased endocardial wall stress in failing human hearts.

Gelsolin is a cardiodependent actin filament severing protein that can control many aspects of actin organisation, such as severing the fast growing end of each actin

filament and nucleating actin assembly (Yin, 1987). The importance of gelsolin regulation has previously been studied in heart failure, where it is likely that increased gelsolin expression may contribute to the perturbation of the thin filament organisation and altered calcium channel regulation (Lader *et al.*, 1999). However, it has not previously been investigated in relation to transmural wall stress.

In the normal adult heart, brain natriuretic peptide (BNP) is expressed at low levels in both the atrium and ventricles. However, in the diseased state it is highly upregulated and plasma BNP levels have been shown to correlate with LV dysfunction (Muders *et al.*, 1997; McDonagh *et al.*, 1998). In addition, mRNA expression of this gene is often used as an index for ventricular dysfunction in CHF. Atrial natriuretic peptide (ANP) is also a well-established marker for the early diagnosis of LV dysfunction. It is primarily produced by atrial cardiac myocytes and is also upregulated by chronic haemodynamic overload. In this study BNP was chosen as it is more sensitive to stretch than ANP and different amounts of stretch reflect the degree of ventricular overload and stress (Mantymaa *et al.*, 1993). In addition, BNP was incorporated into this study as a reference gene because a previous investigation demonstrated a transmural difference in mRNA expression, where it was highly upregulated, predominately in the endocardium of the failing human ventricle (Prestle *et al.*, 1999).

It is now known that the expression of multiple classes of genes are altered in human heart failure, some of which provide compensatory mechanisms to sustain cardiac function and some which contribute to the development of heart failure (Yang *et al.*, 2000). However, few studies have investigated the transmural expression of these genes. The aim of this study was to investigate whether there was a difference in gene expression of the ET system and cytoskeletal proteins across the wall of human failing LV myocardium and to identify if these alterations related to transmural differences in wall stress.

6.2 Methods

Myocardial tissue was obtained from the Scottish Cardiac Transplant unit between October 1997 and February 1998. Left ventricular (LV) epicardial (epi) and endocardial (endo) samples of explanted hearts were used from 6 patients with end-stage heart failure undergoing cardiac transplantation. Endocardial samples were obtained from trabeculated regions of muscle lying within the ventricular cavity. Epicardial samples were dissected from a region of the ventricular wall immediately under the visceral pericardial fat. Samples were immediately frozen in liquid nitrogen and stored at -70°C until use. Age, aetiology, haemodynamic data and medication are listed in *Table 6.1*.

Total mRNA isolation and synthesis of first strand cDNA were carried out as described in section 2.3. Gene specific primers were designed as previously discussed for BNP, prepro ET-1 (ppET-1), the ET_A receptor, the ET_B receptor and gelsolin (section 2.3.5.2). Titin N2BA and N2B each contain unique sequences within the I band region (Freiburg *et al.*, 2000) and primers were specifically designed to amplify products within these sequences for the respective isoforms (section 2.3.5.2). Primers for GAPDH were also designed to serve as an external control.

Template cDNA was amplified using polymerase chain reaction (PCR) (see section 2.3.5.4) and myocardial mRNA levels of BNP, ppET-1, ET_A receptor, ET_B receptor, gelsolin, titin N2BA and titin N2B were quantified by densitometry and expressed as a ratio to GAPDH (section 2.3.6). Two-fold serial dilutions of cDNA template were prepared and amplified by PCR to verify the changes observed in ratios of gene-of-interest:GAPDH (section 2.3.5.5).

Table 6.1. Clinical and haemodynamic data of patients with heart failure

Patient	Sex	Age	Etiology	PCWP (mmHg)	EF (%)	PAP (mmHg)	CO	Medication
Subject 1	M	59	HCM	28	14	46/25	na	Ca, Di, Fr, As, Be
Subject 2	M	56	IHD	26	12	40/23	2.3	Li, Bu, Wa, Di
Subject 3	M	49	IHD	30	4	52/31	na	Di, Wa, Li, Am, Bu, Lo, Is
Subject 4	M	46	IDCM	38	11	55/36	na	Li, Bu, Wa, Di, Be
Subject 5	M	39	ConHD	27	12	33/21	1.68	Ra, Fr, Di
Subject 6	M	53	IDCM	28	15	50/30	1.1	Ca, Di, Be, Fr, As

HCM indicates hypertrophic cardiomyopathy; IHD, ischaemic heart disease; IDCM, idiopathic dilated cardiomyopathy; ConHD, congenital heart disease; PCWP, pulmonary capillary wedge pressure (mmHg); EF, ejection fraction; PAP, pulmonary artery pressure (mmHg); CO, cardiac output ($L \cdot min^{-1}$); Ca, captopril; Di, digoxin, Fr, frusemide; As, aspirin; Be, bendrofluazide; Li, Lisinopril; Bu, bumetanide; Wa, warfarin; Am, amiodarone; Lo, omeprazole; Is, isosorbide mononitrate; Ra, ramipril; na, not available.

6.3 Results

Transmural gene expression of the ET system, cytoskeletal proteins and the hypertrophic marker BNP were evaluated in failing human LV myocardium using a semi-quantitative RT-PCR method. *Figure 6.1* shows scanned agarose gels of the genes-of-interest, BNP, ppET-1, the ET_A receptor, the ET_B receptor, gelsolin, N2A titin and N2B titin. mRNA levels of the constitutively expressed enzyme GAPDH were also examined to quantify the gene-of-interest: GAPDH ratio.

It can be seen in *Figure 6.1* that the abundance of GAPDH is relatively uniform irrespective of aetiology or whether the band represents an epi or endocardial sample. Expression of GAPDH mRNA following amplification of serial dilutions of template also provided similar densitometric results between groups (*Figure 6.2*), confirming that this semi-quantitative RT-PCR method was reliable.

In contrast to GAPDH mRNA expression, the gel illustrating cardiac BNP mRNA tended to have brighter bands most consistently for endocardial samples (*Figure 6.1*). This observation was confirmed by examination of the BNP: GAPDH ratio from end-stage heart failure tissue (*Figure 6.3*), however, different aetiologies displayed different degrees of expression (*Figure 6.4*). BNP mRNA levels across the wall were not significantly different in tissues from subject 1 (HCM), however all other subjects demonstrated BNP mRNA to be most abundant in the endocardium. Subjects 4 and 6, the IDCM pathology, exhibited a large endocardial expression of BNP mRNA (~4-fold greater) with respect to the epicardial expression (*Figure 6.4*) This was also evidenced by the serial dilution curves for this aetiology (*Figure 6.5*).

ppET-1 mRNA: GAPDH mRNA ratio exhibited a similar distribution to that of the BNP: GAPDH ratio, where the majority of cases showed that ppET-1: GAPDH ratio was most abundant in the endocardial tissue samples of hearts from patients with end-stage heart failure (*Figures 6.3 and 6.4*).

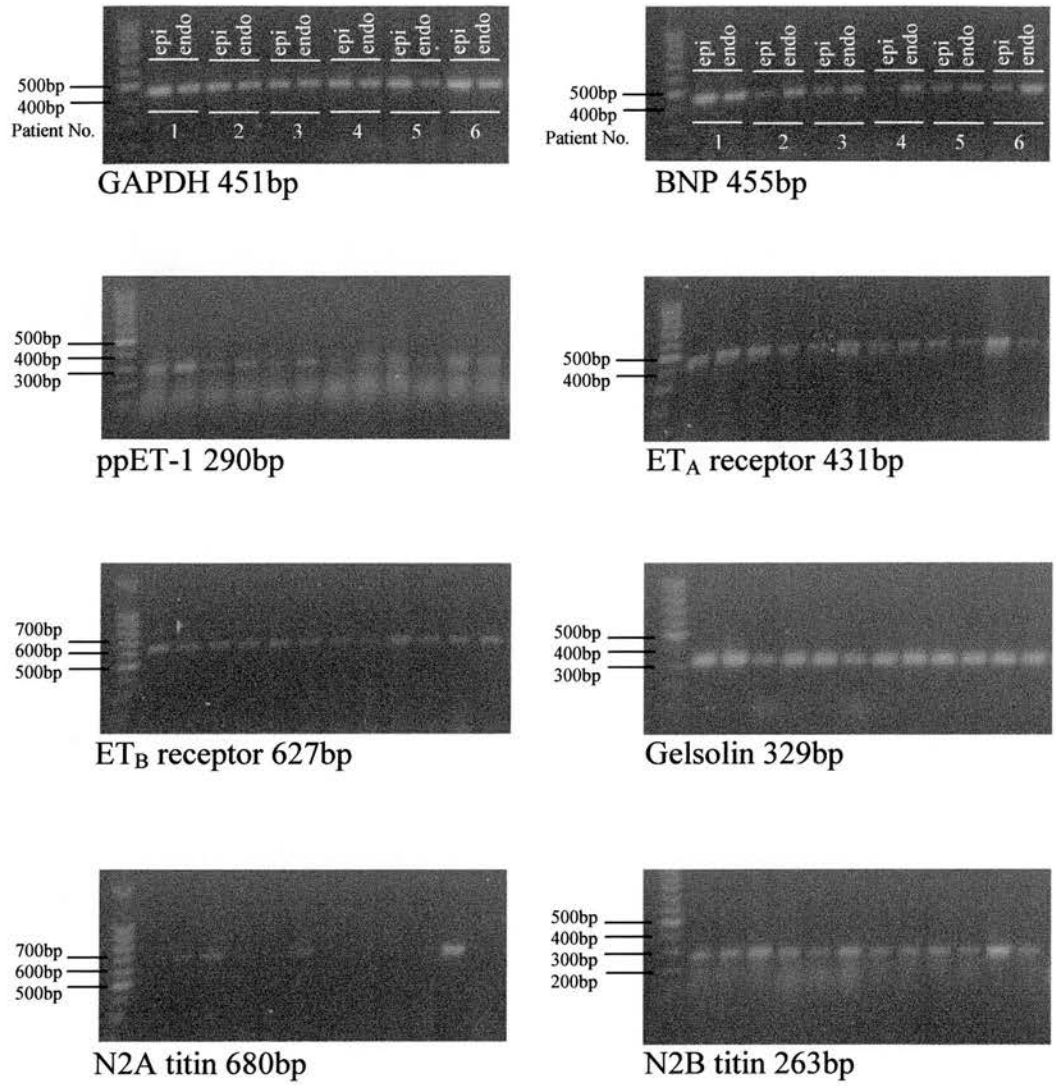


Figure 6.1. Representative agarose gels showing transmural mRNA levels of GAPDH, BNP, ppET-1, ET_A receptor, ET_B receptor, gelsolin, N2A titin and N2B titin in epicardium (epi) and endocardium (endo) of the left ventricular free wall from 6 end-stage failing hearts.

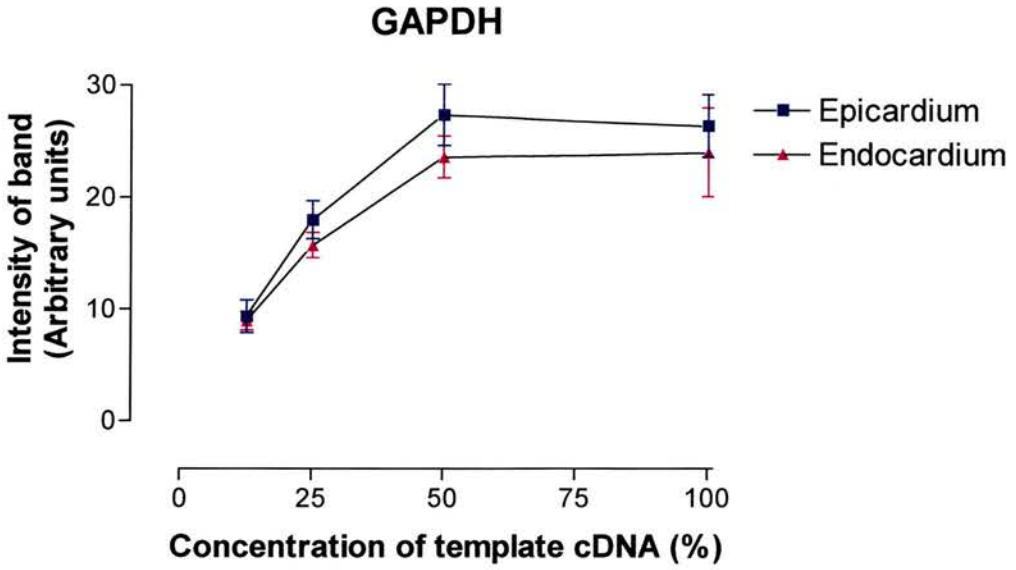


Figure 6.2. The densitometric values of transmural mRNA expression of the ubiquitous housekeeping gene GAPDH following 2-fold serial dilution of cDNA template from patients with end-stage heart failure of various aetiologies (n=6).

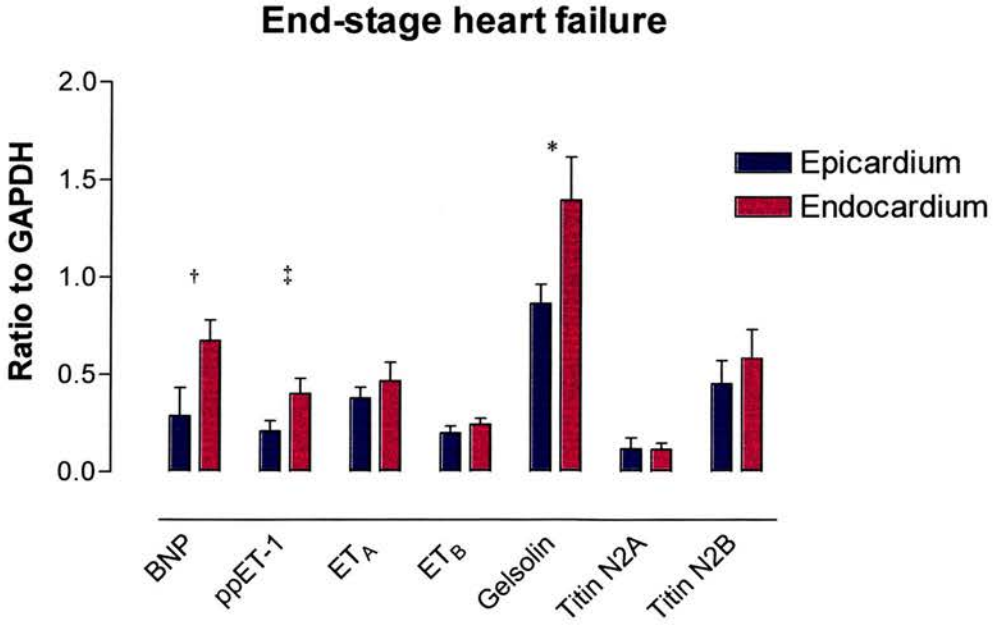
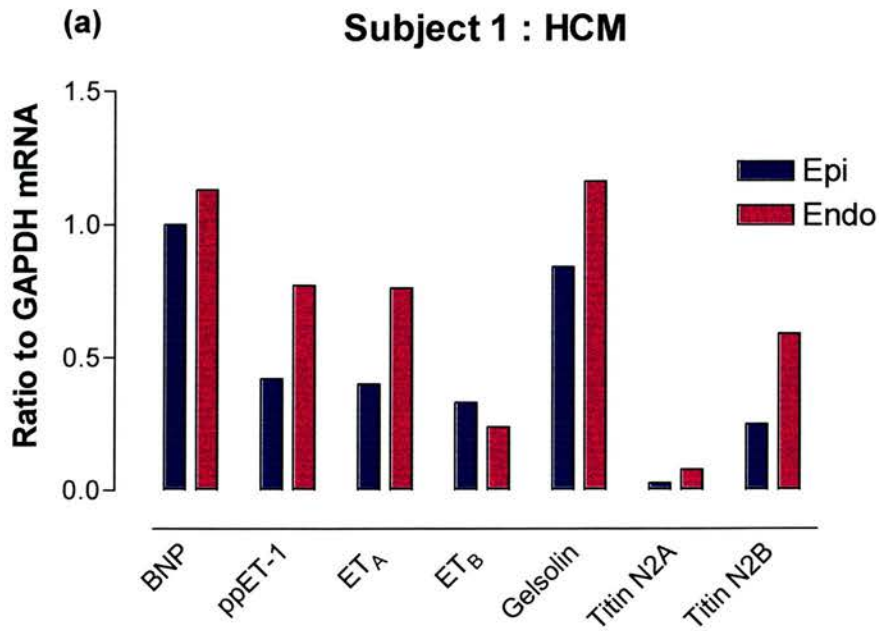
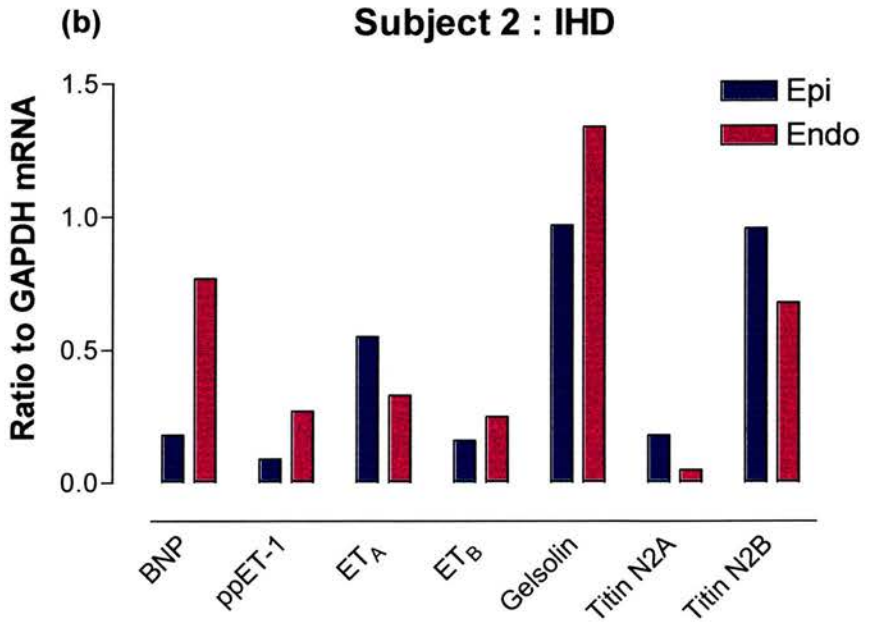
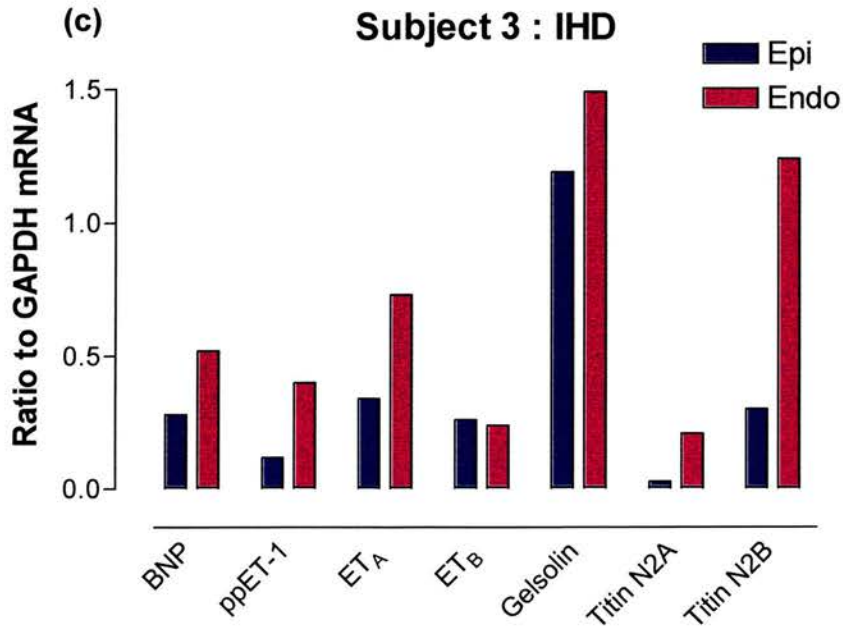
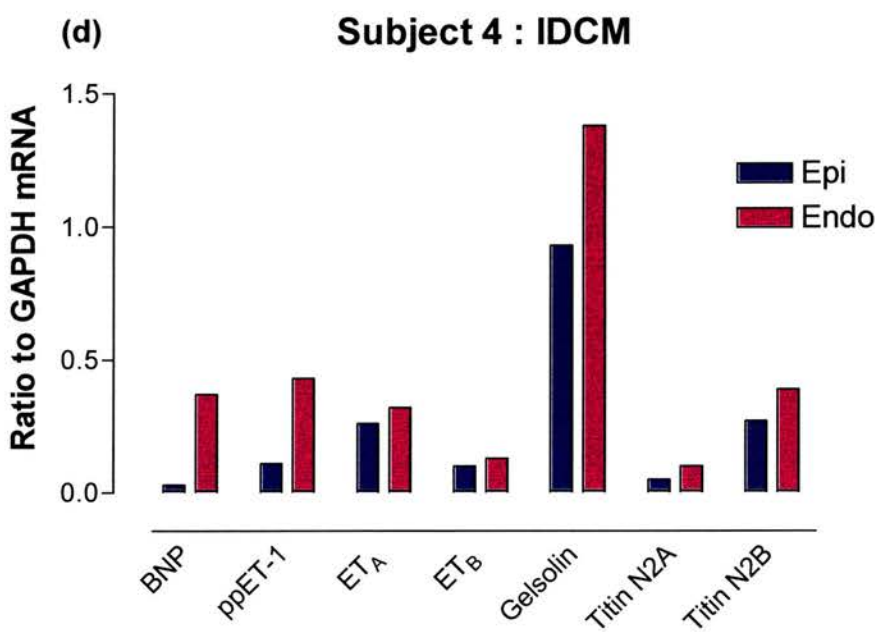


*Figure 6.3. The ratio of genes-of-interest:GAPDH mRNA in epi- and endocardial samples from explanted end-stage heart failure human myocardium (n=6), † P=0.06, ‡ P=0.07, * P=0.05.*


(a)
Subject 1 : HCM
Ratio to GAPDH mRNA
1.5
1.0
0.5
0.0
Epi
Endo
BNP
ppET-1
ET$_A$
ET$_B$
Gelsolin
Titin N2A
Titin N2B


(b)
Subject 2 : IHD
Ratio to GAPDH mRNA
1.5
1.0
0.5
0.0
Epi
Endo
BNP
ppET-1
ET$_A$
ET$_B$
Gelsolin
Titin N2A
Titin N2B


(c)
Subject 3 : IHD
Epi
Endo
Ratio to GAPDH mRNA
1.5
1.0
0.5
0.0
BNP
ppET-1
ET$_A$
ET$_B$
Gelsolin
Titin N2A
Titin N2B


(d)
Subject 4 : IDCM
Epi
Endo
Ratio to GAPDH mRNA
1.5
1.0
0.5
0.0
BNP
ppET-1
ET$_A$
ET$_B$
Gelsolin
Titin N2A
Titin N2B

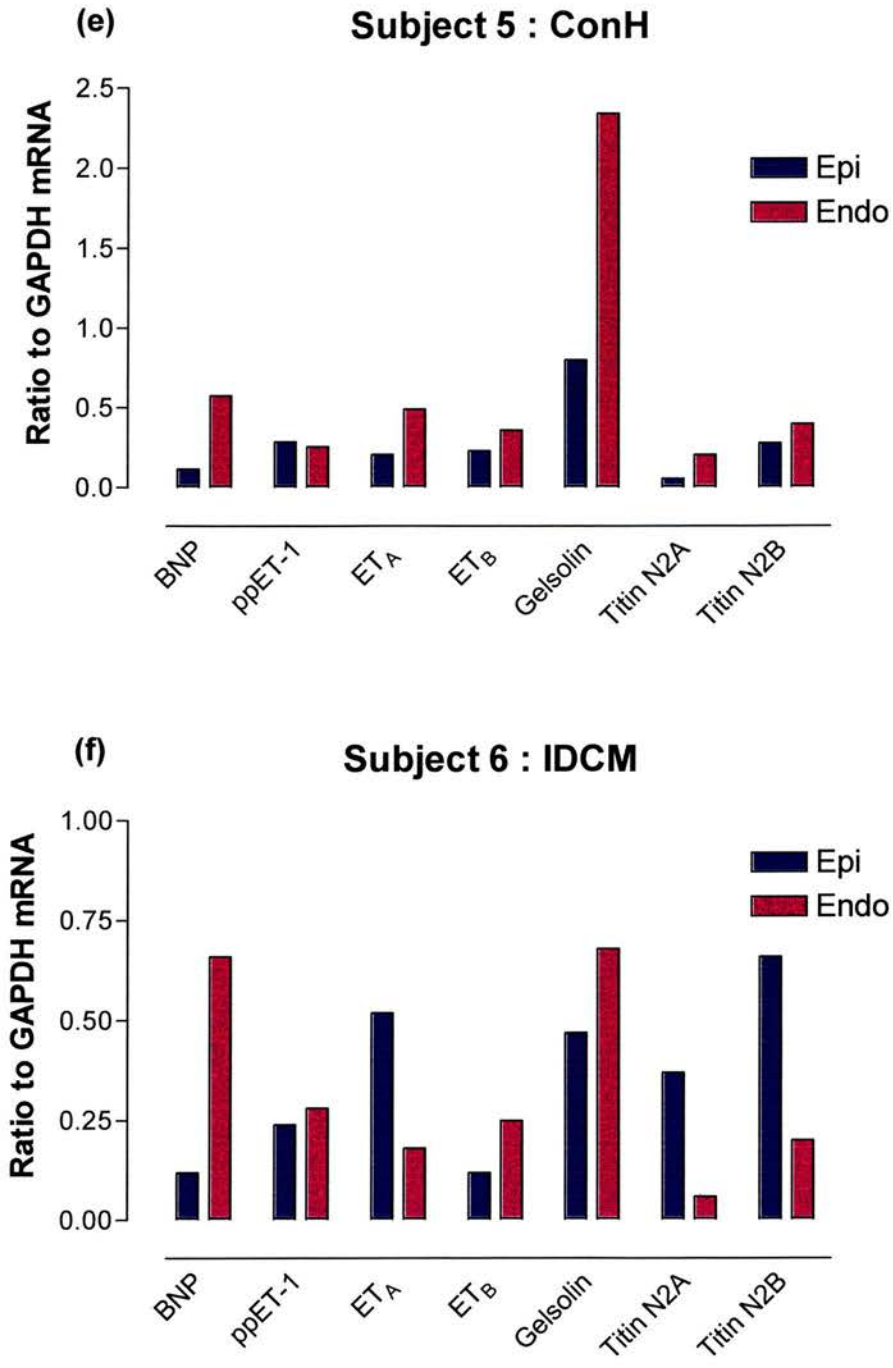


Figure 6.4. The transmural difference of mRNA expression levels of BNP, ppET-1, the ET_A receptor, the ET_B receptor, gelsolin and the titin N2A and N2B isoforms, normalised per GAPDH mRNA level in epi- and endocardial samples of the LV wall of 6 patients (a-f) with end-stage failing heart failure of different aetiologies.

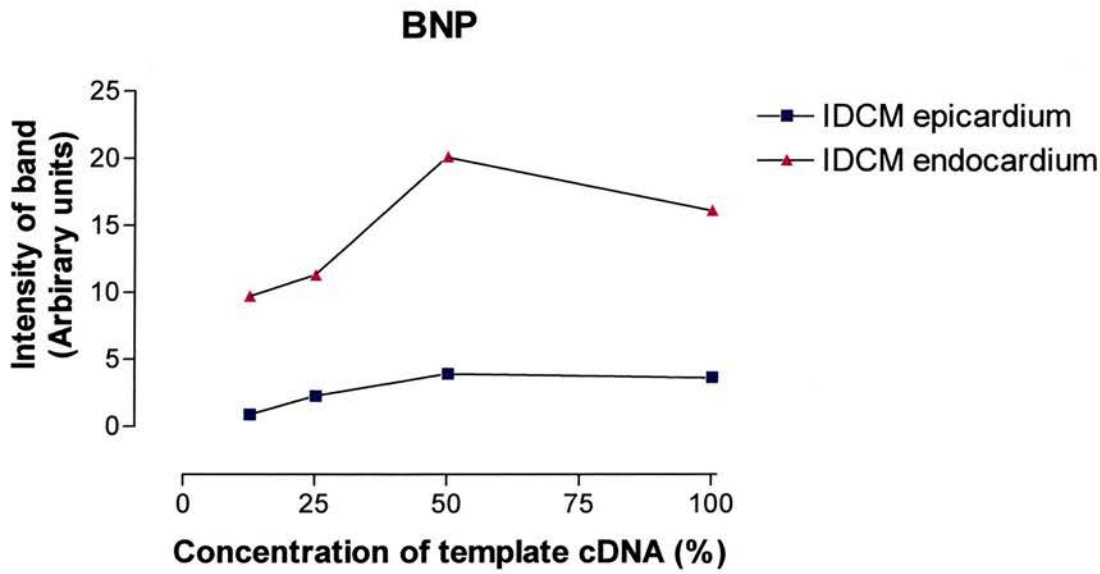


Figure 6.5. Densitometric transmural expression of brain natriuretic peptide (BNP) mRNA following 2-fold serial dilution of cDNA template from hearts of patients with idiopathic dilated cardiomyopathy (IDCM) (n=2).

ET_A receptor mRNA expression as a ratio to GAPDH mRNA was more varied although it tended to be higher in the endocardial samples of LV myocardium from patients with end-stage heart failure (*Figures 6.3*). This was clear in subjects 1 (HCM), 3 (IHD), 4 (IDCM) and 5 (ConHD) (*Figure 6.4*), however, in subjects 2 (IHD) and 6 (IDCM), the ET_A: GAPDH mRNA ratio was greater in the epicardium and this was also identified in the serial dilution curves (*Figure 6.6*). These patients did not display the same aetiology but were of a similar age and had similar haemodynamics (*Table 6.1*). The ET_B receptor: GAPDH mRNA ratio also had the tendency to be higher in the endocardial layer, rather than the epicardial layer but no major pattern emerged (*Figures 6.3 and 6.4*).

The gelsolin: GAPDH mRNA ratio was consistently more abundant in the endocardial sample of each subject compared to the epicardial sample (*Figure 6.4*). This difference was also reflected in the serial dilution of the template cDNA (*Figure 6.7*). Both titin N2A: GAPDH and titin N2B: GAPDH mRNA ratios follow a similar distribution pattern. Most subjects exhibited a greater titin: GAPDH ratio in the endocardial samples, while subjects 2 (IHD) and 6 (IDCM) were the exception, expressing abundance in the epicardium (*Figure 6.4*). The two different expression patterns of the titin isofoms did not reflect the aetiology of the subjects but did match that of the ET_A: GAPDH mRNA pattern (*Figure 6.4*). Serial dilution curves also confirmed the tendency towards abundance in these samples (*Figure 6.8*).

Figure 6.9 shows summary diagrams of the two different patterns of the ET_A receptor, titin N2A and N2B: GAPDH mRNA expression ratios. Subjects 1, 3, 4 and 5 expressed a greater ratio in the endocardial samples while subjects 2 and 6 expressed ET_A, titin N2A and titin N2B: GAPDH mRNA to be greater in the epicardium of hearts from patients with end-stage heart failure. These findings were not related to aetiology.

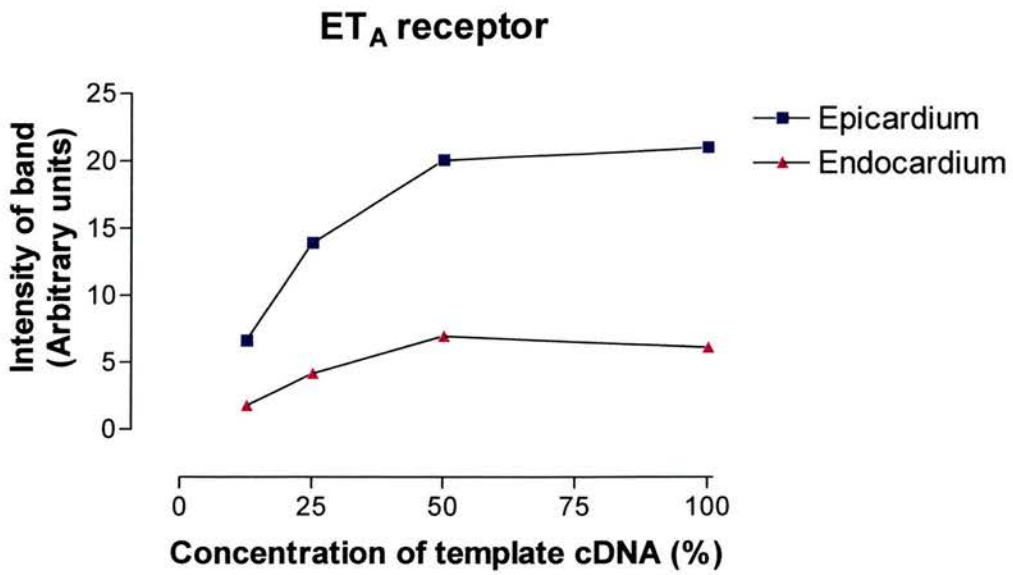


Figure 6.6. The densitometric values of transmural mRNA expression of the ET_A receptor following 2-fold serial dilution of the cDNA template from patients with end-stage heart failure of various aetiologies (n=2).

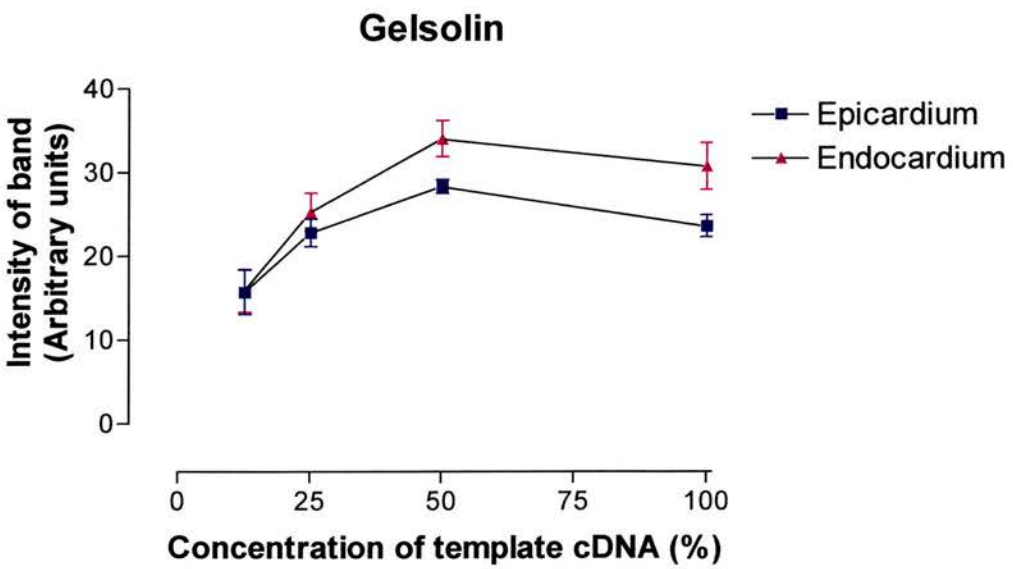


Figure 6.7. Densitometric values of transmural gelsolin mRNA expression following 2-fold serial dilution of the template cDNA from patients with end-stage heart failure (n=6).

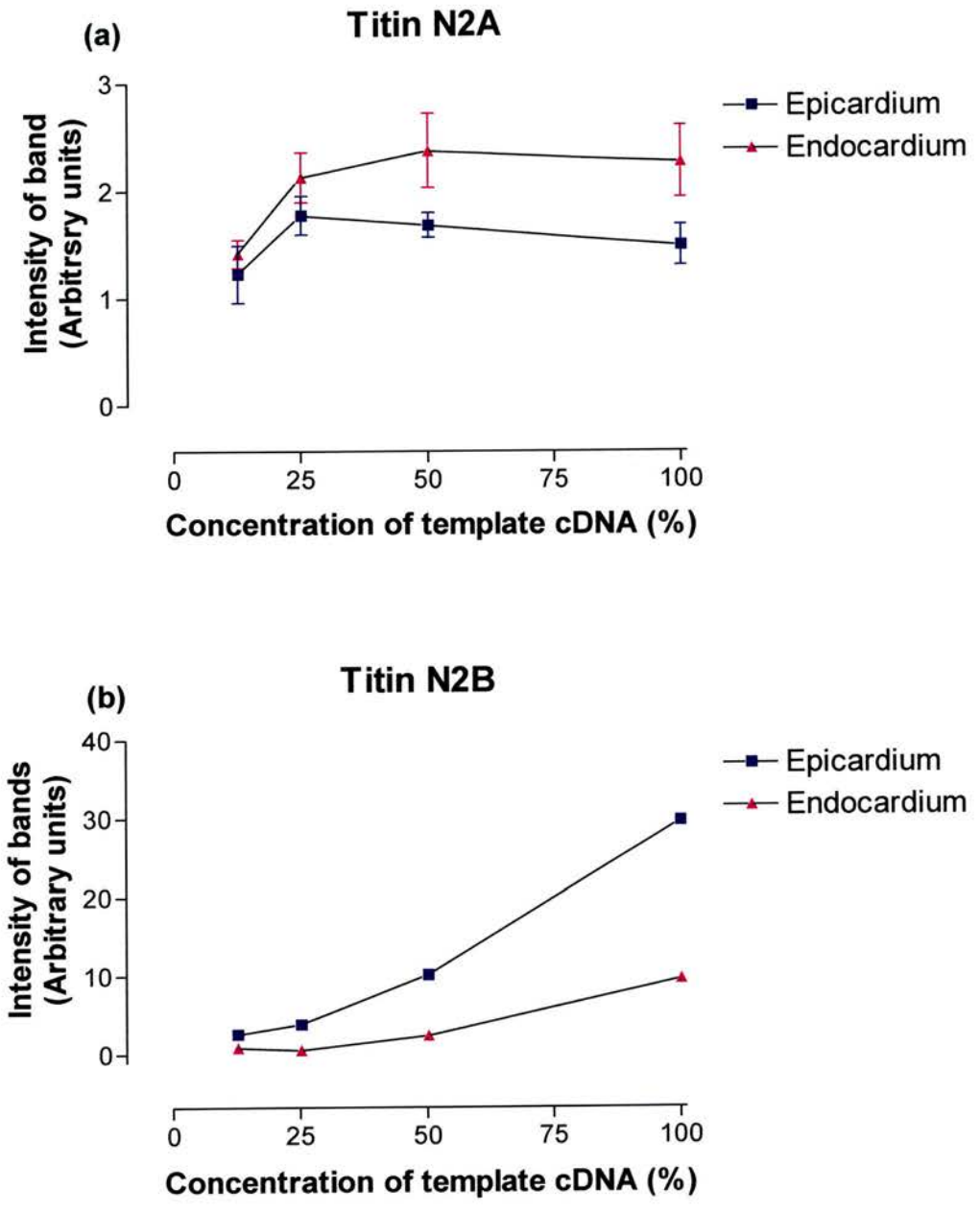


Figure 6.8. The densitometric values of transmural mRNA expression of (a) titin N2A (n=4) and (b) titin N2B (n=2), showing the different patterns of expression following 2-fold serial dilution of the cDNA template from patients with end-stage heart failure of various aetiologies.

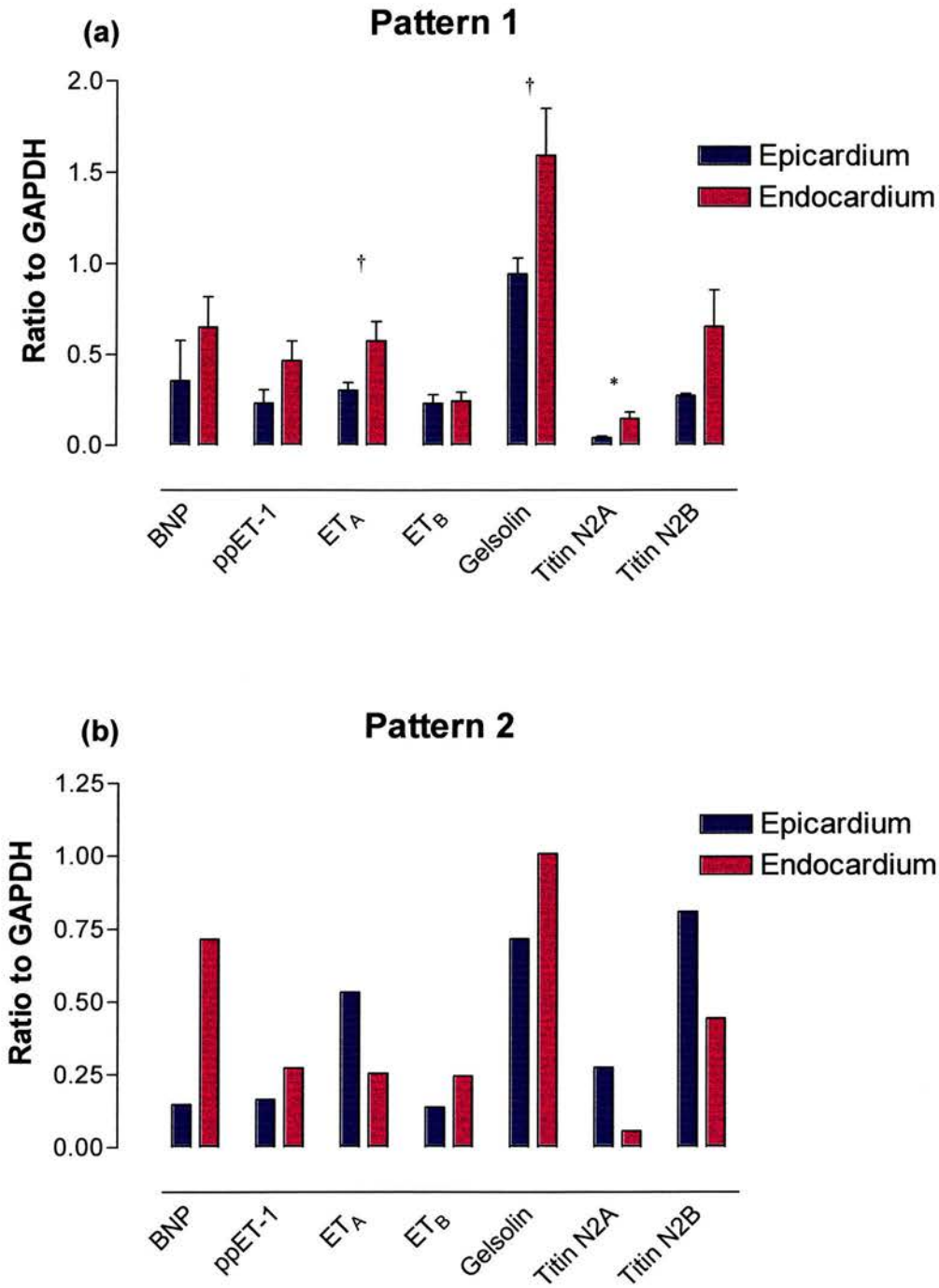


*Figure 6.9. The two different transmural patterns of distribution of the ET_A receptor, titin N2A and titin N2B: GAPDH mRNA ratio found in LV myocardial samples from patients with end-stage heart failure. (a) pattern 1, where the gene ratio is elevated in the endocardial section (n=4) †P=0.05, *P<0.05 and (b) pattern 2, where the genes tend to be more abundant in the endocardium (n=2).*

6.4 Discussion

The purpose of this study was to investigate whether the expression of eight different genes differed across the LV wall of hearts from patients with end-stage heart failure and to establish whether the alterations related to ventricular wall stress.

Previous studies have identified that in a transmural cross-section, cardiac wall stress is highest in the endocardial layer (Mirsky, 1973; Yin, 1981). In the normal heart, under normal loading conditions, ventricular expression of natriuretic peptides is low. However, in heart failure, a disproportionate increase of ventricular radius relative to wall thickness, results in increased ventricular wall stress and this is considered to be a major determinant for ventricular secretion of natriuretic peptides (Kinnunen *et al.*, 1993; Ruskoaho *et al.*, 1997). In the current study, a distinct transmural difference of BNP: GAPDH mRNA ratio existed across the LV wall. Semi-quantitative RT-PCR evaluation of mRNA expression levels showed that the hypertrophic marker was more abundant in the endocardial layer of hearts from patients with end-stage heart failure. These findings are consistent with Prestle *et al.* (1999), who demonstrated that BNP mRNA was highly upregulated, predominantly in the endocardium compared to the epicardium of failing human LV myocardium.

As previously discussed, studies have indicated a role for ET-1 in the pathophysiology of heart failure (McMurray *et al.*, 1992; Pacher *et al.*, 1996). Elevated production of ET-1 is thought to contribute to systemic and pulmonary vasoconstriction, as well as to cardiac hypertrophy, fibrosis and remodelling (Cooper, 1997; Mulder *et al.*, 1997). Elevation of ppET-1 mRNA has previously been demonstrated in human heart failure, however, it does appear to be related to aetiology. Zolk *et al.* (1999) failed to detect a change in ppET-1 mRNA expression in hearts from patients with end-stage chronic heart failure (CHF) due to DCM compared to non-failing. In contrast, Serneri *et al.* (1999) demonstrated that patients with adequate hypertrophy compared to inadequate hypertrophy showed higher values of cardiac formation of ET-1 and Big ET-1 as well as increased ppET-1 mRNA levels. Furthermore, this selective stimulus for ET-1 activation was also observed by Serneri *et al.* (2000), where ppET-1 mRNA levels were found to be

significantly increased in the myocardium and isolated myocytes from ischaemic cardiomyopathy (ICM) compared to DCM hearts. However, although ischaemia has been reported to induce ET-1 formation (Tønnessen *et al.*, 1995; Ito *et al.*, 1996), there is an ischaemic component present in the heart of DCM patients (Parodi *et al.*, 1993; Beltrami *et al.*, 1995), suggesting that pressure overload is a major mechanism of cardiac ET-1 system activation in ICM (Serneri *et al.*, 2000). In the current study, the expression ratio of ppET-1: GAPDH mRNA was not compared to normal human LV myocardium, as none was available. However, expression was more pronounced in the endocardial layer of human failing LV, suggesting that like BNP, regulation of ppET-1 is determined by ventricular wall stress. In addition, expression of ppET-1 may also have been influenced by BNP (see section 1.2.3).

Previous studies of the cardiac ET-1 system in heart failure have only evaluated the differences between failing and non-failing tissues. In the current study, transmural mRNA levels of ET_A and ET_B receptor mRNA were examined to establish whether receptor expression was related to the altered wall stresses known to occur in heart failure. The current study identified that expression of the ET_A receptor across the LV wall of hearts from patients with end-stage heart failure displayed two different patterns. Distribution in 4 of the 6 hearts studied exhibited a greater abundance of ET_A: GAPDH mRNA ratio in the endocardial sections as opposed to the epicardium, which were not related to aetiology. However, this pattern did coincide with the distribution of the titin isoforms. Expression of the ET_B receptor: GAPDH mRNA ratio in the current study did not follow any particular pattern although it had the tendency to be higher in the endocardial layer, rather than the epicardial layer. It is known that the ET_B receptor is less abundant than the ET_A receptor in the human LV (Pönicke *et al.*, 1998), therefore, it is not surprising that it is less sensitive to change across the wall. BNP: GAPDH mRNA expression tended to have a greater difference across the wall in pattern 2 compared to pattern 1, however, sample size would have to been increased for these observations to be statistically significant. Gelsolin: GAPDH mRNA expression was also elevated in the endocardium but was to a similar extent in both distribution patterns.

ET-1 receptor subtypes have been investigated in myocardial homogenates of hearts from patients with end-stage DCM (Pönicke *et al.*, 1998; Pieske *et al.*, 1999; Zolk *et al.*, 1999) or ICM (Pönicke *et al.*, 1998) with conflicting results. Serneri *et al.* (2000) compared the two types, DCM and ICM directly and found that although plasma levels of ET-1 were elevated in both types of heart failure, consistent differences in the expression pattern of the cardiac ET-1 receptors existed between LV samples of end-stage failing hearts from ICM or DCM patients (Serneri *et al.*, 2000). Cardiac ET-1 formation, mRNA levels for ppET-1 and the ET_A and ET_B receptors were increased in both the myocardium and isolated myocytes of ICM compared to DCM hearts. ET-1 binding studies also indicated that both ET_A and ET_B receptors were increased in ICM but not DCM LV and this selective upregulation of the ET-1 system components in ICM patients appears to be functionally important in the maintenance of cardiac function (Serneri *et al.*, 2000). In the current study transmural samples were evaluated without the reference of non-failing myocardium. However, the lack of a distinct pattern of ET receptor expression across the wall relating to aetiology suggests that changes are subjective to other influences such as severity or degree of hypertrophy. Additionally, Asano *et al.* (2002) recently demonstrated that both ET_A and ET_B receptor mRNA were increased in DCM and ICM failing human hearts, while ET_B receptor mRNA was only increased in ICM. The study also suggested that based on the lack of correlation with ET-1 tissue levels, it was unlikely that the failure-related changes in mRNA and protein levels of the ET_A and ET_B receptors resulted from homogenous regulation by agonist exposure (Asano *et al.*, 2002).

As with most of the genes investigated in this study, the expression of the actin filament severing protein, gelsolin has been evaluated in human heart failure but has not been assessed transmurally. Yang *et al.* (2000), using high-density oligonucleotide arrays, previously identified that gelsolin mRNA levels were upregulated (2-3 fold) in LV tissue from patients with end-stage heart failure. These changes correlated with similar changes observed in cardiomyopathic hearts from 2 different lines of transgenic mice exhibiting cardiac hypertrophy (rac1-expressing mice) or heart failure phenotypes (tropomodulin-overexpressing mice) (Yang *et al.*,

2000) and in coronary artery ligated compared to sham-operated rats (Stanton *et al.*, 2000). In the current study, the gelsolin: GAPDH mRNA ratio was consistently upregulated in the endocardial sample of hearts from patients with end-stage heart failure. Considering that over expression of gelsolin leads to disorganisation of the thin actin filaments (Lader *et al.*, 1999) and thereby functional impairment, the altered transmural expression is consistent with the transmural changes in LV wall stress that occur in heart failure.

In addition to gelsolin, a gene responsible for cell structure and motility, Yang *et al.* (2000) and Stanton *et al.* (2000) demonstrated that multiple classes of genes were altered in heart failure, such as cytoskeletal and myofibrillar genes, genes involved in metabolism and genes encoding stress proteins. Furthermore, Tan *et al.* (2002) have since demonstrated that certain gene clusters found within heart failure groups, associate with specific aetiologies, suggesting that heart failure of different aetiologies may involve different genetic determinants for their development. However, in the present study, transmural expression did not appear to relate to the aetiology of heart failure.

The elastic behaviour of the sarcomeric protein titin is responsible for the generation of the restoring forces in cardiomyocytes. Studies have previously shown that the myocardium co-expresses two distinct titin isoforms, N2A and N2B transcripts and that the N2B isoform is the stiffer of the two (Labeit & Kolmerer, 1995). The expression ratio of these has been found to vary greatly between species, between the atria and ventricles and within different locations of the pig and dog hearts (Cazorla *et al.*, 2000; Bell *et al.*, 2000; Trombitás *et al.*, 2001) and is a means of modulating cardiac stiffness. Cell-cell stiffness variation within species is related to strain equalisation of muscle fibres in the different layers of the wall. Consistent with this, transmural expression of the titin isoforms in the current study of hearts from patient with end-stage heart failure demonstrated an increased N2B: GAPDH mRNA ratio in the endocardium of 4 of the 6 hearts studied. In addition, the expression ratio of N2A: GAPDH mRNA also followed this distribution pattern, as did the ET_A receptor (discussed above) but was not related to aetiology. However, knowing that the

different isoforms influence the compliance of the cardiac muscle, these results indicate that the stiffness of the endocardial layer in pattern one was much greater than in the epicardium, correlating with elevations of endocardial wall stress as evidenced by elevated the endocardial BNP: GAPDH mRNA ratio.

Previous studies of titin isoforms in heart failure have demonstrated that titin N2B is upregulated in the LV wall of dogs with tachycardia-induced DCM (Wu *et al.*, 2002). In contrast, Neagoe *et al.* (2002) reported that the titin N2BA: N2B ratio was altered in the LV of patients with coronary artery disease (CAD), compared to nonischaemic human LV samples. The relative N2BA titin content was greater in the CAD hearts than the normal hearts and was associated with lower myofibrillar passive tension. The reduction of N2B titin also had a profound effect on mechanical heart function as evidenced by abnormally high left ventricular end-diastolic pressure (Neagoe *et al.*, 2002). In addition, Collins *et al.* (1996) previously showed that titin transcripts were increased in compensated LV pressure overload hypertrophy but declined after the transition to decompensated congestive HF. Similarly, Warren *et al.* (2003) demonstrated that expression of titin N2B was increased, while titin N2BA decreased in response to pressure overload in hypertensive rats. Interestingly, recent studies have identified that mutations within the cardiac-specific N2B region of the titin gene were associated with CHF due to familial DCM (Itoh-Satoh *et al.*, 2002; Gerull *et al.*, 2002). Together, these studies suggest that different regulatory mechanisms control the entry to either the N2B or N2BA splice pathways, thereby controlling the extensibility and perhaps adding to ventricular mechanical stress in heart failure. It has also been suggested that the switch of titin isoforms in heart failure could impair the ability of the heart to use the Frank-Starling mechanism (Sutko *et al.*, 2001), however, in the present study, the N2B distribution pattern did not appear to correlate with cardiac output or any of the other haemodynamic parameters measured.

In summary, investigation of transmural gene expression in the current study demonstrated that most genes were more abundant in the endocardial sections of hearts from patients with end-stage heart failure. As expected, the BNP: GAPDH

mRNA ratio was consistently greater in the endocardial layer, compared to the epicardium, as were the expression ratios for ppET-1 and gelsolin: GAPDH. This distribution is most likely attributed to and/or partly responsible for the difference in ventricular wall stress, which is known to occur across the LV wall of failing hearts. However, the components of the ET-1 system did not show consistent transmural expression as the expression of the ET-1 receptors differed across the wall. In most subjects, the ET_B receptor was most abundant in the endocardium, although no particular pattern emerged, possibly because the ET_B receptor is generally less abundant in cardiac muscle. In contrast, the ET_A receptor: GAPDH mRNA ratio was greater in the endocardium of 4 of the 6 hearts studied. This differential expression followed that of the titin isoforms, N2B and N2A, however, the two distribution patterns were unrelated to aetiology. Expression of the N2B isoform is know to be associated with increased stiffness and like the ET_A receptor, is possibly influenced by pressure overload, suggesting that the end-stage failing hearts in pattern 1 exhibited adequate hypertrophy to upregulate the genes in these locations. Some of this variation in transmural gene expression may be due to the different background diagnoses in the patients with one having hypertrophic cardiomyopathy and another congenital heart disease. Increased sample size and comparison with non-failing epi and endocardial tissue would help to clarify the distribution of these genes in various forms of end-stage heart disease, while *in situ* hybridisation studies would specifically identify the location of the titin isoforms. GAPDH mRNA levels were not significantly different across the wall and have previously been shown not to change in response to heart failure (Yang *et al.*, 2000), therefore, its use as an external control was viable, as was the semi-quantitative RT-PCR method which was developed for this study.

Chapter 7

General Discussion

Endothelin-1 (ET-1) has been implicated as playing an important physiological role in cardiovascular regulation and a putative pathophysiological role in a range of diseases. Increased plasma levels and tissue expression of the peptide has been reported in patients with heart failure and correlates well with indices of severity such as pulmonary artery pressure and reduced cardiac index (Pacher *et al.*, 1993).

At the onset of this thesis, there was a distinct lack of clarity regarding the function of cardiac ET receptor subtypes in failing and non-failing myocardium. Studies have suggested that the ET_A receptor is solely responsible for mediating the inotropic responses to ET-1 in isolated cardiomyocytes but function in tissue and organ preparations have been conflicting. Antagonist studies have also been unable to clarify the receptor subtype(s) responsible for cardiac contraction. In addition, studies in our laboratory have revealed a selective upregulation of the ET_B receptor subtype 5-12 weeks post coronary artery ligation (CAL) (Sherry, 2000), the significance of which was unknown. Therefore, the purpose of this study was to better define the expression and function of ET-1 receptors in failing and non-failing rat myocardium *in vivo* and *in vitro*. In addition, ventricular mechanics are altered in pathological states and in diseases characterised by abnormal loading of the heart, normalisation of wall stress appears to govern the rate and extent of hypertrophy. Transmural expression of the genes marking hypertrophy, cytoskeletal genes and components of ET system were examined in relation to elevated left ventricular (LV) wall stress.

In this current PhD investigation, the initial papillary muscle studies indicated, as expected, that contractile function was impaired in rat failing myocardium. However, inotropic responses to the administration of exogenous ET-1 were variable. A slight biphasic response was observed in the sham but not CAL group suggesting that the function of the endothelial ET_B receptor was impaired. LV expression of the ET_B receptor was not significantly altered in the CAL rats 15 weeks post myocardial infarction (MI) compared to the sham-operated rats. This may have been due to a combination of reduced endocardial endothelial ET_B expression and an increased cardiomyocyte ET_B expression, as previous studies in our laboratory have

demonstrated selective ET_B upregulation. The variable contractile responses to exogenous ET-1 were attributed in part to the release of endogenous ET-1 by mechanical stretch. Therefore a follow up study was designed using selective and dual ET-1 receptor antagonists so that the roles of the receptor subtypes might be better defined in heart failure.

The antagonist approach was tested using right ventricular papillary muscles from normal rats, with the intention of following up in CAL rats, to assess the contribution of endogenous ET-1 to basal myocardial contractility. The findings from this antagonist study indicated that both ET receptors contributed to the inotropic response in normal rat myocardium and that ET-1 was released by mechanical stretch of the papillary muscle. In addition, responses to exogenous calcium were altered following incubation with ET-1 receptor antagonists, suggesting that calcium handling may be an important site of ET-1 action. However, significance levels were not quite reached, therefore the protocol was repeated in a second set of control rats to increase sample size. The second antagonist study was conducted 6 months after the initial investigation but the results were not as expected. In contrast to the previous results, ET-1 receptor antagonists did not influence inotropic responses of papillary muscles in study 2. This outcome was confusing, a possible explanation for these findings was that the antagonist was perhaps unable to penetrate the tissue for some reason, or unable to block all receptors. In order to find some sort of answer, mRNA expression of the ET system and the hypertrophic marker, atrial natriuretic peptide (ANP) were investigated. The results indicated that there was a difference in gene expression between the two studies, which were carried out 6 months apart. Seasonal differences in gene expression are not uncommon and could have been responsible for the different responses observed between the two studies. Therefore, due to the inconsistent responses achieved using this preparation, it was decided not to continue with an antagonist study in CAL rats as it was not guaranteed to provide a satisfactory conclusion.

In vivo assessment of cardiac contractility by echocardiography enabled the study of real time cardiac function without any tissue manipulation or complications such as

stretch released ET-1. In the CAL versus sham study, the results quite clearly showed the effect of LV dysfunction on the end-systolic pressure-dimension relationship, confirming the observed reduction of ejection fraction, shortening fraction and elevation of the LV end-diastolic pressure. In order to better determine the influence of endogenous ET-1 on cardiac contractility in normal animals, a protocol was designed whereby bosentan, a dual ET_A/ET_B receptor antagonist was administered chronically for 1 week. When compared to a placebo group, no significant haemodynamic or contractile influence was observed, suggesting that endogenous ET-1 did not play a central role in cardiac contractility. However, it is likely that as the animals were healthy, other factors may have compensated for the loss of ET-1 mediated tone.

In contrast, acute administration of bosentan significantly reduced the contractility of the heart, shifting the pressure-dimension loop of the cardiac cycle down and to the left. This may have been a direct effect via cardiac ET-1 receptors or may have been due to reduced peripheral resistance, thereby reducing the afterload/preload to act via the Frank-Starling mechanism, or most likely a combination of both. It would be interesting to repeat this bosentan study using CAL and sham-operated rats to investigate whether intervention with dual ET_A/ET_B receptor blockade at different time points (acute, chronic or delayed chronic) affected the contractility of the myocardium as studies of LV pressure suggest that it could.

Long-term treatment with mixed ET_A/ET_B receptor antagonists has previously been shown to improve survival and has beneficial effects on the haemodynamics of rats with chronic heart failure (CHF) (Mulder *et al.*, 1997; Sakai *et al.*, 2000). In addition, Clozel *et al.* (2002) recently reported that acute administration of tezosentan, another dual ET_A/ET_B receptor antagonist, 1 or 24 hours post MI was found to improve long-term survival of CHF rats by causing a decrease of pulmonary oedema and preventing cardiac hypertrophy. However, not all studies have demonstrated such positive outcomes. Use of the mixed ET_A/ET_B receptor antagonist LU420627 resulted in reduced survival, increased LV dilatation and cardiac dysfunction whether the treatment was started early or late after MI, indicating unfavourable actions of the

drug independent of timing of treatment after MI (Nguyen *et al.*, 2001). In addition, another recent study reported the adverse effects of early intervention with SB209670, a dual ET_A/ET_B receptor antagonist, when treatment was initiated 48 hours after MI in rats (Øie *et al.*, 2002).

Prompted by the early positive results from studies in animal models of heart failure and from acute studies in patients, it was logical to expect that chronic blockade of ET receptors might be of benefit to patients with CHF. However, since commencing this PhD investigation, the clinical trials of ET receptor antagonists in heart failure have mainly been negative. Early studies with bosentan were disappointing due to the excess of adverse effects encountered and some small clinical trials were even terminated early. Treatment with bosentan has been associated with the early development of worsening heart failure, perhaps due to the high dose of drug used (Cowburn & Cleland, 2001; van Veldhuisen & Poole-Wilson, 2001). In addition, a further concern is the excess of abnormal liver function tests which have been found in bosentan-treated groups (van Veldhuisen & Poole-Wilson, 2001). Two larger studies using lower doses of bosentan have also produced disappointing results. ENABLE 1 and ENABLE 2 (Endothelin Antagonist Bosentan for Lowering Cardiac Events) studies found that long-term treatment with the dual receptor antagonist did not alter mortality or hospitalisation of patients with severe heart failure. It seemed that fluid retention was a major contributory factor to this outcome, even despite an increased use of diuretics. The negative results of the ENABLE studies have added to doubts over the potential benefits of non-selective ET-1 receptor blockade in heart failure. However, further dose-response studies may still determine an optimal dosing strategy for specific aetiologies. In addition, administration of bosentan has in acute studies been shown to improve the haemodynamic parameters in CHF patients (Sutsch *et al.*, 1998). Furthermore, Torre-Amione *et al.* (2001) demonstrated that acute administration of tezosentan in patients with moderate to severe heart failure led to an improved haemodynamic profile and was safe and well tolerated. Therefore it may be the case that timing is important. Bosentan is currently used successfully in the treatment of pulmonary arterial hypertension although its use requires close monitoring due to its potential of causing liver dysfunction and its teratogenic effects

(as reviewed by Cheng, 2003). Clozel *et al.* (personal communication) have also indicated that administration of bosentan in acute heart failure is of benefit and it is likely that the ET antagonist may have an application here.

The complexity of the ET system and its diverse roles in multiple disease states ensures that it will continue to be targeted for drug development. Therefore selective ET_A receptor antagonism may provide certain benefits. In a recent study, Luscher *et al.*, (2002) (HEAT; Heart Failure ET_A Receptor Blockade Trial) demonstrated that 3 weeks treatment with darusentan, selective ET_A receptor antagonist, improved the cardiac index of patients with CHF. However, again it was highlighted that dosage was important as higher doses were associated with a trend to more adverse events. Long-term randomised clinical trials of selective antagonists have still to be conducted and compared to dual ET_A/ET_B receptor blockade to establish whether selective ET_A blockade is beneficial over dual ET receptor blockade in human CHF.

A myriad of genes undergo altered expression during the development of heart failure (Yang *et al.*, 2000; Stanton *et al.*, 2000). In this PhD investigation expression of genes associated with hypertrophy, cytoskeletal proteins and components of the ET system were investigated across the LV wall of explanted hearts from patients with end-stage heart failure of various aetiologies to establish if they were related to wall stress. As expected the hypertrophic marker was more abundant in the endocardium, the layer associated with increased wall stress. In addition, gelsolin, a regulator of cytoskeletal organisation also displayed greater expression in the endocardium as did the ET-1 precursor prepro-ET-1. The ET receptors did not follow any particular pattern in relation to aetiology but the ET_A receptor did display the same expression pattern as titin, the giant molecule which contributes to the elasticity of the myocardium. Most types of heart failure contain hypertrophic myocardium however, the extent and distribution will depend on local wall stress therefore various regions might contain more hypertrophic markers than others. Variability of gene expression across the wall may therefore depend on the area from which the epi and endocardial samples were taken and may not necessarily reflect the distribution of expression of genes in the whole myocardium. To follow up these findings it

would be interesting to carry out immunohistochemical analysis on sections of the tissues, looking at the titin isoforms and the ET system to see how they are distributed within the different layers of different aetiologies and how they associated to varying levels stiffness. In addition if more samples were available and some from non-failing human tissue, the comparison may lead to a better understanding of the ET system in different types of heart failure and the changes which occur within the wall organisation.

Finally, as previously discussed (see section 1.2.5), generation of knock-out mice has demonstrated the importance of the ET system in development. Although many of the phenotypes are lethal, generation of new conditional or specific knock-outs, such as the cardiac specific ET_B receptor knock-out currently under characterisation in our laboratory, may provide further insight into the complexity of the ET system in the failing and non-failing myocardium.

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